THE AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE

Sustained since 1926 *by the Endowment of Sir Joseph Cooke Verco for the publication of Medical Research*

MANAGING EDITORS:

J. B. CLELAND (ADELAIDE), C. STANTON HICKS (ADELAIDE).

Assisted in the selection of papers for publication by the following

BOARD OF EDITORS:

W. E. AGAR (Melbourne); L. B. BULL (Adelaide); H. G. CHAPMAN (Sydney); R. CILENTO (Brisbane); A. J. EWART (Melbourne); T. HARVEY JOHNSTON (Adelaide); C. H. KELLAWAY (Melbourne); J. McLUCKIE (Sydney); A. E. MILLS (Sydney); T. G. B. OSBORN (Sydney); W. A. OSBORNE (Melbourne); W. J. PENFOLD (Melbourne); J. A. PRESCOTT (Adelaide); W. RAY (Adelaide); A. E. V. RICHARDSON (Adelaide); N. D. ROYLE (Sydney); F. P. SANDES (Sydney); H. A. WOODRUFF (Melbourne); H. J. WILKINSON (Adelaide); W. J. YOUNG (Melbourne).

VOL. VII

PUBLISHED BY THE UNIVERSITY OF ADELAIDE

MANAGED FOR THE UNIVERSITY BY THE MEDICAL SCIENCES CLUB OF SOUTH AUSTRALIA

PRINTED AT THE HASSELL PRESS, ADELAIDE

1930

UNIVERSITY OF
MINNESOTA
LIBRARY

CONTENTS

PARTS 1 AND 2. 16TH JUNE, 1930.

	PAGE
Obituary	1
JOAN H. NORRIS: "A Chemical Investigation in Victoria (Australia) of the Blood of Cattle and Sheep"	3
A. W. TURNER and D. MURNANE: "On the Presence of the Non-Pathogenic *Trypanosoma Melophagium* in the Blood of Victorian Sheep, and its transmission by *Melophagus Ovinus*"	5
A. W. TURNER and D. MURNANE: "A Preliminary Note on the Occurrence of *Trypanosoma Theileri* in the Blood of Cattle in Victoria"	9
HENRY FRANCIS HOLDEN and MAVIS FREEMAN: "Experiments on Denatured Proteins"	13
F. M. BURNET: "A Physical Difference Amongst Bacteriophages" ..	27
A. E. COATES and O. W. TIEGS: "Further Observations on Certain Alleged Effects of Sympathetic Nerves on Skeletal Muscle" ..	37
EMILY M. DAY and ADOLPH BOLLIGER: "On a Simplified Method for the Determination of Blood Cholesterol"	41
GEOFFREY H. SMITH: "Factors Affecting the Deposition of Dental Calculus"	45
J. BURTON CLELAND: "Further Results in Blood Grouping Central Australian Aborigines"	79
GWENDOLYN M. CHENEY: "Black-Heart of Apricots in Victoria" ..	91

627478

PART 3. 16TH SEPTEMBER, 1930.

PAGE

T. HARVEY JOHNSTON: "The Anatomy of the Trematode, *Macrophyllida Antarctica* (Hughes)" 101

T. HARVEY JOHNSTON: "A New Species of Trematode of the Genus Anoplodiscus" 108

LANCE S. WALTERS: "The Colorimetric Estimation of Nitrogen by 'Direct Nesslerization,' with a Note on a Modified Nessler-Folin Reagent" 113

MAVIS FREEMAN: "The Action of Dilute Formaldehyde Solution on Proteins and Protein Derivatives" 117

DONALD F. THOMSON: "Observations on the Venom of a Large Australian Snake, *Pseudechis Australis* (Gray). 1. Synonymy" .. 125

C. H. KELLAWAY and DONALD F. THOMSON: "Observations on the Venom of a Large Australian Snake, *Pseudechis Australis* (Gray). 2. The Venom Yields and Venom" 134

PART 4. 16TH DECEMBER, 1930.

E. S. HORNING and I. D. MILLER: "Chromidial Extrusion, and its Relationship to Atypical Nuclear Phenomena in Tumour Cells" .. 151

DOROTHY DIXON: "The Micro-Organisms of the Tertiary Red Sands near Melbourne, Victoria" 161

C. STANTON HICKS: "The Ultra-Violet Absorption Spectra of the Quinoline Group" 171

F. M. BURNET and MARGOT McKIE: "Balanced Salt Action as manifested in Bacteriophage Phenomena" 183

F. M. BURNET and MARGOT McKIE: "The Electrical Behaviour of Bacteriophages" 199

FLORENCE McCOY HILL: "The Identity of the 'Primitive Cell'" .. 211

H. C. GURNEY: "The Inheritance and Linkage Relations of Xantha Seedlings in Maize" 215

WILLIAM ALEXANDER OSBORNE: "The Toxicity of Blood which has been Frozen" 223

WILLIAM ALEXANDER OSBORNE: "The Action on Mammalian Circulation of the Skin Secretion of Hyla Aurea" 226

Thorburn Brailsford Robertson

Born March 18, 1886. Died January 18, 1930.

The tragically sudden death of Professor Brailsford Robertson, after only a few days of illness, has removed from our midst an outstanding personality, has robbed the scientific world of a leading investigator, and has deprived Australia of the benefits of his ripe experience and mature judgment. As the prime mover in the founding of this journal, and as its principal Editor since its formation, his loss, so unexpected and so sudden, has fallen with particular incidence upon us. Though it is intended, on a later occasion, to publish a full appreciation of him, including a bibliography of his writings, the Board of Editors cannot let this occasion pass without adding their personal appreciation of the eminent scientific qualities of their late colleague, of his indomitable capacity for work, of his good nature, of his thoroughness, and of his broadness of mind.

A CHEMICAL INVESTIGATION IN VICTORIA (AUSTRALIA) OF THE BLOOD OF CATTLE AND SHEEP

by

JOAN H. NORRIS

(From the Biochemistry Department, University of Melbourne).

(Submitted for publication 15th April, 1930.)

To obtain as full a knowledge as possible of the amounts of the chemical constituents of the blood of cattle and sheep, the work started last year (1) has been continued to include values for total acid-soluble phosphate and iron in the blood of cattle.

For comparison the inorganic phosphate has again been estimated by Fiske and Subbarow's method, the organic phosphate by their method also (2), but with a slight modification. 5 cc. of the protein-free filtrate, using trichloracetic acid as precipitant, were gently boiled with 5 cc. of 5N. sulphuric acid until the flask was filled with fumes and the mixture was dark brown; a drop of nitric acid cleared the solution, which after reboiling for about a minute was allowed to cool. 15 cc. of distilled water were then added, and the mixture reduced, by boiling, to between 5 cc. and 10 cc. The flask was cooled under running water, its contents washed into a 50 cc. standard flask, and treated with molybdate and 1.2.4. aminonaphthol sulphonic acid, as described by Fiske and Subbarow.

The iron was estimated by the colorimetric method of San Yin Wong (3). The haemoglobin molecule is broken up by conc. H_2SO_4 aided by potassium persulphate, which sets free the iron. Tungstic acid is used to precipitate the proteins, the colour being developed with potassium thiocyanate in an aliquot part of the filtrate, and matched against a standard solution of an iron salt.

FURTHER ANALYSES OF BLOOD OF CATTLE.

STEERS.

Inorg. Phos. mgm/100cc.	Total Phos. mgm/100cc.	Iron. mgm/100cc.	Sample No.
4·57	9·17	—	67
4·47	9·84	—	68
3·67	10·96	57·45	72
3·72	9·79	56·20	73

Inorg. Phos. mgm/100cc.	Total Phos. mgm/100cc.	Iron. mgm/100cc.	Sample No.
5·16	11·55	54·05	74
3·90	9·25	35·10	75
4·57	12·11	58·80	76
5·33	12·11	64·10	77
4·10	10·93	64·10	78
5·23	11·42	64·50	83
5·92	11·42	64·50	84
3·52	9·58	60·60	85
5·92	12·22	63·35	86
6·40	10·12	63·30	87
4·88	8·00	63·70	88
4·60	9·75	64·50	89
4·28	10·32	59·50	96
2·57	7·92	54·65	101
3·33	8·00	64·90	102
4·44	8·94	56·80	103
3·67	8·79	68·00	107
3·36	8·60	66·65	108
6·56	13·34	—	109
3·48	9·14	—	112
4·12	11·76	—	113
3·29	8·42	—	114
4·44	10·13	60·54	Mean
	COWS.		
8·25	12·54	—	69
5·88	11·10	—	70
4·23	10·53	55·55	71
—	9·53	—	79
—	11·42	—	80
—	11·68	—	81
—	13·17	—	82
6·78	15·69	75·75	90
5·33	9·09	59·85	91
5·16	17·39	68·00	92
3·86	14·42	51·80	93
6·67	14·95	61·35	94
6·91	16·50	58·15	95
7·62	16·50	64·50	97
3·62	12·90	53·45	98
5·16	10·00	54·35	99
4·54	6·10	37·45	100
5·33	12·90	56·15	104
6·84	11·42	56·80	105
5·80	9·82	68·00	106
7·02	15·54	—	110
7·41	13·79	—	111
6·63	12·59	57·92	Mean

REFERENCES.

1. Norris and Chamberlin: Aust. Journ. Exper. Biol. and Med. Sci., 6 (1929), p. 285.
2. Fiske and Subbarow: Journ. Biol. Chem., 66 (1925), p. 375.
3. San Yin Wong: Journ. Biol. Chem., 67 (1928), p. 409.

ON THE PRESENCE OF THE NON-PATHOGENIC *TRYPANOSOMA MELOPHAGIUM* IN THE BLOOD OF VICTORIAN SHEEP, AND ITS TRANSMISSION BY *MELOPHAGUS OVINUS*

by

A. W. TURNER AND D. MURNANE

(Council for Scientific and Industrial Research, Australia)

(From the Veterinary Research Institute, University of Melbourne.)

(Submitted for publication 15th April, 1930.)

The non-pathogenic sheep trypanosome, *Trypanosoma melophagium*, Flu, 1908, was first seen by accident in a blood smear by Woodcock (1910) in England. It was subsequently recorded for the first time in Germany by Behn (1911), in Holland and Douwes (1920), in Russia by Boshenko and Zeiss (1928), and in Argentina by Dios (1928). It appears to be quite harmless, and is usually extremely rare in the blood, so that a large number of blood smears may be examined before a single specimen is found.

In 1905, in Germany, Pfeiffer first described in the gut of the sheep-ked *Melophagus ovinus* L. the flagellate subsequently known as *Crithidia melophagia*. They are usually present in enormous numbers, and are very widespread among keds, in which they appear to be harmless. They have since been reported in many parts of the world, but apparently not hitherto in Australia. Nöller's comparative cultural studies (1919) led him strongly to suggest that the ked flagellate is the crithidial form of the sheep trypanosome, while Kleine (1919) showed that non-infected keds become infected with crithidia only after feeding on sheep harbouring trypanosomes in their blood. It was not until 1923 that the identity of the two forms was established by Hoare, who showed that the method of infection of sheep is not by inoculation, but by ingestion. According to his investigations the ked is hatched out non-infected; as a result of the ingestion of the blood of sheep infected with trypanosomes the posterior portion of its gut becomes heavily infested with crithidia. Non-infected sheep become infected by crushing and swallowing keds in an effort to allay the irritation caused by their bites. The infection is not directly transmissible from ked to ked. Hoare's work has not hitherto been confirmed.

Melophagus ovinus is present in all States of the Commonwealth. It therefore appeared likely to us that local sheep and keds would be affected naturally

with the trypanosome. Examination of keds collected from sheep at the Institute indeed showed an enormous infection in the posterior three-fourths or two-thirds of the intestine, as described by Hoare. Pieces of gut were teased in normal saline and examined under a coverslip. Smears of this emulsion were stained by Leishman, Giemsa, or Heidenhain's iron haematoxylin. In sections of keds stained by the last method the whole surface of the epithelial cells appeared to be thickly carpeted with a layer of flagellates. We have been able to culture the flagellates in blood-broth in a few instances.

Turning next to laboratory sheep infested with keds, we examined the blood, but found, as have other workers, that the trypanosomes are very rare. We have never seen them in ordinary thin blood smears, but have been able to obtain them by centrifuging laked, defibrinated blood, as recommended by Teague and Clark (1918) for *Trypanosoma theileri* in cattle. (See the following article on trypanosomiasis of cattle in this issue.) By this method the trypanosomes are concentrated. They are extremely motile when examined fresh. The sediment may be smeared out and either fixed with osmic acid vapour or air dried and subsequently stained with Leishman or Giemsa. The osmic acid fixation preserves the flagellum and undulating membrane very well.

As regards the transmission of the trypanosome we have been able to confirm Hoare's work. Ked-free lambs raised from birth in the laboratory, and kept away from infected sheep, have remained free from both keds and trypanosomes. Such lambs, fed with an emulsion of the ground-up abdomens of some hundreds of infected keds, have shown trypanosomes in blood cultures from the sixth day onwards after feeding. Generally speaking the trypanosomes, even after artificial infection, have been rare. For example, Hoare examined a flock of 50 sheep heavily infested with keds. From each sheep a blood culture and several thin and thick blood films were taken. By the cultural method the presence of the trypanosomes in about 80% of the sheep was established, although he was unable to find any specimens in the blood films, of which at least 300 were examined. Even during the course of his examination of over 1,000 preparations of blood from five artificially infected sheep only six trypanosomes were found in ordinary thin dried films and "several more living ones in fresh blood examined under a coverslip."

With regard to natural infection we have examined ten flock sheep by the microscopic method. Five which were heavily ked-infested all showed trypanosomes after defibrinating, laking, and centrifuging the blood. Of the five remaining, which were lightly ked-infested, three showed trypanosomes in defibrinated, laked, centrifuged blood. In the case of naturally infected flock sheep it has occasionally been necessary to repeat the examination once or twice before the presence of the trypanosomes was confirmed.

Recent work by various investigators has suggested that the spleen may be the organ responsible for the destruction of trypanosomes in the blood (Nieschultz and Wawa-Roentoe, 1929; Bruynoghe and Vassiliadis, 1929). The

point seems to be disputed, but it appeared to us worthy of trial. Accordingly a non-infected and ked-free lamb was splenectomized, and about ten weeks later was fed with an emulsion of the abdomens of 598 keds, the feeding extending over four days. Commencing from the eighth day after the first feed, trypanosomes were found in fresh defibrinated blood in such numbers as to make their detection even in ordinary thin blood smears possible, and certainly in much greater numbers than in non-splenectomized sheep examined by us or described by other workers. In our splenectomized, artificially infected lamb we have seen two or three per preparation of fresh blood under a coverslip, and up to ten living trypanosomes in a drop of the deposit from defibrinated, laked, centrifuged blood, and have had no difficulty in getting dozens of specimens stained for photographs. Hoare, on the other hand, in his 1,300 preparations from non-splenectomized sheep, obtained only three trypanosomes suitable for examination.

While the results on a single lamb are not at all conclusive, it certainly appears that previous splenectomy has had some influence on the degree of infection. However, we have never seen any dividing forms indicative of multiplication in the body.

We also tried the effect of splenectomy on a lamb which at one time had given a positive blood culture as a result of the ingestion of infected keds, and in which the infection later apparently died out. The splenectomy in this case failed to cause a reappearance of the trypanosomes in the blood-stream. We have now under way experiments to ascertain whether splenectomy of known infected lambs will give rise to a marked increase in the number of trypanosomes in the blood.

As regards culturing the trypanosome we have had very poor results up to the present. Only in two instances, after incubating at 30°C., as recommended by both Nöller and Hoare, have we obtained cultures—once after seven days and once after a lapse of three weeks. The reason for this is not clear, but similar difficulties have been met with by other workers.

Finally we have applied the formol-gel test, advocated by Cernaianu (1928) in the diagnosis of dourine, to the sera of sheep harbouring the trypanosome, but without success. Only in the case of an old sheep in poor condition did gelification occur.

Description of the Trypanosome.

Trypanosoma melophagium is one of the larger of mammalian trypanosomes. Its size is variable, some forms being longer and narrower than others.

It has a definite oval nucleus near the middle of the body, and the kinetoplast begins a short distance behind it. From there the flagellum passes on the edge of the undulating membrane to the anterior end of the body, often being prolonged as a free flagellum. Both ends of the body are sharply drawn out. The following measurements were made from representative specimens obtained by laking and centrifuging defibrinated blood from a splenectomized lamb artifi-

cially infected nine weeks previously. The preparations were fixed with osmic acid vapour and stained with Giemsa.

Measurements are given in microns.

	A	B	C	D
Total length from posterior extremity of body to tip of flagellum ..	40·75	48·25	50·25	53·75
Posterior extremity of body to kinetoplast	10	16	15·5	17
Kinetoplast to posterior border of nucleus	9	4	5	6·75
Dimensions of nucleus	2·75 × 1·75	2·75 × 1·75	2·5 × 1·5	2·75 × 2
Anterior border of nucleus to anterior extremity of body	19	19·5	20·75	22
Free flagellum	5	6	4	2·5
Width of body (not including membrane) at level of nucleus ..	2·75	2·25	2·5	2·75

CONCLUSIONS.

1. *Trypanosoma melophagium* has been found in sheep in Victoria.
2. Its transmission by *Melophagus ovinus* by ingestion has been confirmed.
3. Pre-infectional splenectomy appears to increase the number of circulating trypanosomes.

REFERENCES.

1. Behn, P.: Berl. tierärzt. Woch., 27 (1911), p. 768.
2. Boshenko, B. and Zeiss, H.: Rév. de Microbiol. Epidémiol et Parasit., 7 (1928), p. 461; Abstr. in Trop. Vet. Bull., 17 (1929), p. 114.
3. Bruynoghe, R. and Vassiliadis, P.: Ann. Soc. Belge de Méd. Trop., 9 (1929), p. 191.
4. Cernaianu, C.: Compt. Rend. Soc. Biol., 99 (1928), p. 1181.
5. Dios, L.: Compt. Rend. Soc. Biol., 99 (1928), p. 1502.
6. Douwes, J.: Deutsche tierärzt. Woch., 28 (1920), p. 553.
7. Hoare, C.: Vet. Journ., 79 (1923), p. 271.
8. Hoare, C.: Parasitology, 15 (1923), p. 365.
9. Kleine, F.: Deutsche tierärzt. Woch., 27 (1919), p. 408.
10. Nieschultz, O. and Wawa-Roentoe, F.: Tijd. voor Diergeneeskunde, 56 (1929), afl. 21.
11. Nöller, W.: Arch. f. Schiffs- u. Trop.-Hyg., 21 (1919), p. 53.
12. Nöller, W.: Deutsche tierärzt. Woch., 27 (1919), p. 327.
13. Nöller, W.: "Die Züchtung der tierischen Parasiten und Krankheitserreger auf künstlichen Nährböden," in Kraus and Uhlenhuth's "Handbuch der mikrobiologischen Technik" (Berlin und Wien), 1 (1923), p. 647.
14. Pfeiffer, E.: Zeitschr. f. Hyg. u. Infktskr., 50 (1905), p. 324.
15. Teague, O. and Clarke, H.: Journ. Inf. Dis., 22 (1918), p. 154.
16. Woodcock, H.: Quart. Journ. Micr. Sci., 55 (1910), p. 641.

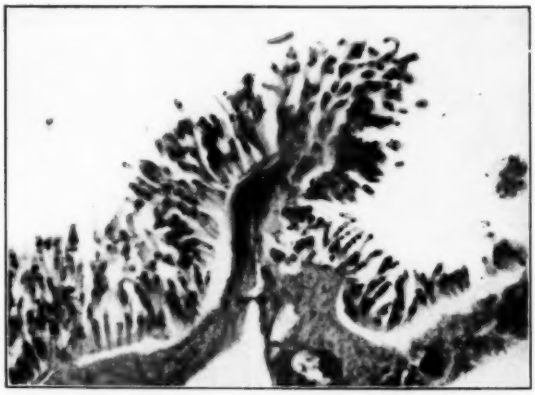


Figure 1. Transverse section of the gut of *Melophagus ovinus*, showing the intense infection by the erithidial phase of *Trypanosoma melophagium* (natural infection). Fixed by Debell's modification of Bouin's Piero-formol; paraffin section; stained with Heidenham's iron haematoxylin. × 1,000.

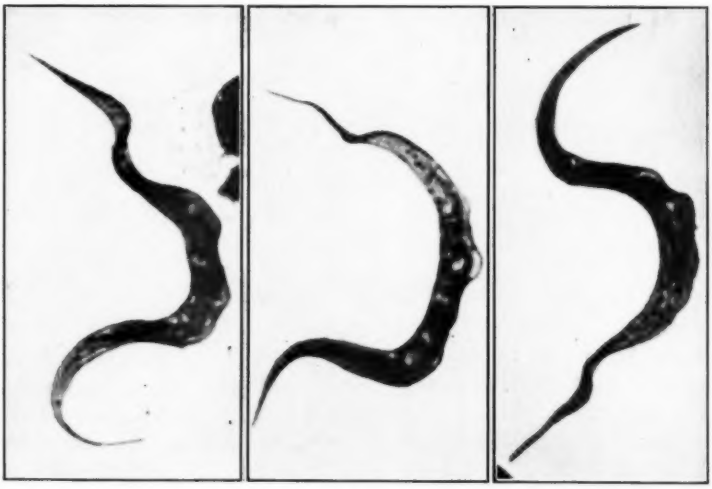


Figure 2. *Trypanosoma melophagium* from laked and centrifuged defibrinated blood of a splenectomized lamb artificially infected by feeding on infected keds nine weeks previously. Fixed by osmic acid vapour; stained with Giemsa. × 2,000.

A PRELIMINARY NOTE ON THE OCCURRENCE OF *TRYPANOSOMA THEILERI* IN THE BLOOD OF CATTLE IN VICTORIA

by

A. W. TURNER AND D. MURNANE

(Council for Scientific and Industrial Research, Australia)

(From the Veterinary Research Institute, University of Melbourne.)

(Submitted for publication 15th April, 1930.)

In 1903 Theiler, in the Transvaal, described in the blood of cattle a large trypanosome, which was later recognized to be non-pathogenic or only weakly pathogenic. Laveran, having examined Theiler's smears in 1902, had named it *Trypanosoma theileri*. Under various names, of which only *Trypanosoma theileri* is now regarded as valid, it has been recorded in many parts of the world, including Somaliland, Algiers, Tunis, England, France, Denmark, Sweden, Russia, Germany, Greece, India, Singapore, Annam, Japan, the Philippines, United States of America, Canada, Panama, and Uruguay; but apparently it has not been recorded before in Australia. It is described by Wenyon (1926) as being about 60–70μ long by 4–5μ wide, though smaller (presumably immature) forms, 25–30μ long, have been reported from many countries. Nevertheless, there does appear to be some variation in the morphology of the trypanosomes of this type, i.e. the large non-pathogenic type, described in various countries. Thus the forms described by Stockman in England were provided with a very well-marked membrane showing three to four distinct undulations.

Under normal circumstances the trypanosome is present in extremely small numbers in the blood, in which case it can be revealed only by cultural methods (Miyajima, 1907, etc.), or by microscopic examination of laked and centrifuged blood (Teague and Clarke, 1918). Exceptionally, however, when the health of the host is affected by intercurrent infections such as East Coast fever (Theiler, 1903), piroplasmosis (Stockman, 1910), rinderpest, surra, or foot and mouth disease (Mayer, 1912), it may become sufficiently numerous to be seen readily in ordinary blood smears.

Theiler (1903) claimed that it is carried by the horse fly, *Hippobosca*, but there seems to be some doubt about this.

Nöller (1925) has shown that in Germany it can be carried by the biting

fly, *Haematopota pluvialis,* in which it exists as a crithidial form. Similar crithidial forms, culturally indistinguishable from *Trypanosoma theileri,* had been reported in the March fly, *Tabanus glaucopis,* in Germany by him in 1916. It is highly probable that tabanids of various species and their allies are the main carriers. The purpose of this note is to record the presence of an apparently non-pathogenic, large trypanosome, morphologically similar to *Trypanosoma theileri,* in the blood of a cow.

Having shown that the sheep trypanosome, *Trypanosoma melophagium,* exists in Australia, we considered it worth while to look for the cattle trypanosome, especially as we had been urged to do so by Sir Arnold Theiler.

For the purpose of a preliminary examination, defibrinated ox blood collected at the abattoirs, was laked with an equal volume of fresh[1] distilled water, and centrifuged well for twenty to thirty minutes. The supernatant fluid was then decanted, and the deposit, consisting mostly of leucocytes and red cell débris, was examined under the microscope. In one case out of twelve bloods examined a few large motile trypanosomes, not so active as *T. melophagium,* were seen. Two specimens were obtained in smeared out deposit, fixed with the vapour of 2% osmic acid solution, and stained with Giemsa.

The following measurements were taken, and are compared with those measured from the published drawings of Lühe. Measurements are given in microns.

	Australian forms.		Lühe's drawings.	
	No. 1	No. 2	a	b
Total length from tip of flagellum to posterior end of body	52·5	66	61·9	67·5
Posterior end of body to kinetoplast ..	9	11	10	12
Kinetoplast to posterior border of nucleus	9	8	7·5	9·5
Dimensions of nucleus	4·5 × 1·75	5 × 2·5	3 × 1·9	3 × 2·5
Anterior border of nucleus to anterior end of body	25	28·5	22·5	22·5
Free flagellum	5	10	15·5	12·5
Breadth of body (not including the undulating membrane)	3·75	4	4·5	3

We have made no attempts yet to cultivate the trypanosome, or to discover the intermediate host in Australia. It may be expected that a careful search for this haemo-flagellate in the smears of ox blood will reveal its presence in a certain percentage of cases, particularly in the more tropical parts of Australia, where piroplasmosis occurs.

[1] Distilled water which has been kept for some weeks often contains various flagellates.

Figure 1. *Trypanosoma theileri* from laked and centrifuged defibrinated blood of a cow (natural infection). Fixed by osmic acid vapours; stained with Giemsa. × 2,000.

REFERENCES.

1. Laveran, A.: Compt. Rend. Acad. Sci., 134 (1902), p. 512.
2. Mayer, M.: "Pathogene Trypanosomen," in von Prowazek's "Handbuch der path. Protozoen" (Leipzig), 1 (1912), p. 299.
3. Miyajima, M.: Philippine Journ. Sci., 2 Med. Sci. (1907), p. 83.
4. Nöller, W.: Berl. tierärzt. Woch., 32 (1916), p. 457.
5. Stockman, S.: Journ. Comp. Path. and Thera., 23 (1910), p. 189.
6. Teague, O. and Clark, H.: Journ. Inf. Dis., 22 (1918), p. 154.
7. Thieler, A.: Journ. Comp. Path. and Thera., 16 (1903), p. 193.
8. Wenyon, C.: "Protozoology" (London), 2 (1926), p. 498.

EXPERIMENTS ON DENATURED PROTEINS

by

HENRY FRANCIS HOLDEN AND MAVIS FREEMAN

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 9th May, 1930.)

This paper describes some experiments on the specific rotation of proteins and the effect of denaturation thereon. The effect of small concentrations of neutral salts on the solubility of denatured proteins near their iso-electric points was examined and comparison made with their effects upon solutions of casein.

While the optical activity of certain native proteins has been closely studied, that of denatured proteins has received less attention, due possibly to the difficulty of preparing solutions suitable for polarimetry. Gamgee and Hill (2) determined in 1904 the optical activity of the "globin" prepared from oxyhaemoglobin by the method of Schulz (10). They found that it had the value [—65·5°], whereas oxyhaemoglobin was slightly dextro-rotary. It is now recognized that this "globin" is a denatured protein. Holden and Freeman (7) found the specific rotation of (native) ox globin to be $[-13°]_{5780}$, which is much lower than that of denatured ox globin $[-72°]_{5780}$. Young (14) found that the optical activity of serum albumin denatured by sunlight is higher than that of the native albumin. It has long been known that the solubility of certain proteins, e.g., serum eu-globulin and casein, is markedly diminished on the acid side of their respective iso-electric points by the presence of neutral salts. Though the mechanism of the effects is obscure, attempts have been made to generalize concerning their course from different points of view.

In 1927 Hewitt (5) published a paper on the optical activity of specimens of several proteins which he considered to be of exceptional purity. Hardy and Gardiner (4) had found that the removal of lipoids from the mixed proteins of serum by precipitation with alcohol and ether had practically no effect on the specific rotation of the mixed protein. Hewitt found that if the proteins, in addition, were extracted with ether the specific rotations of the individual proteins were considerably increased. He also applied this technique to crystalline hen ovalbumin with a like result. It was previously believed, on the authority of Sörensen (11), that hen ovalbumin could not be so treated. Hewitt used the curve of rotatory dispersion of proteins to calculate the position

of the dominant absorption band in the ultra-violet region, and showed that it agreed with the direct observations of other workers. He concluded that the optical activity of the proteins investigated, when lipoids are present, is a variably quantity depending on unknown factors. This would seem to imply that the relationships between proteins and lipoids must be in part that of chemical combination and in part admixture.

The Optical Activity of Denatured Proteins.

To see whether the effect of denaturation on the specific rotation of proteins, suggested by Gamgee and Hill, and observed for serum albumin by Young, held for other proteins several specimens of native proteins were prepared. Portions of these specimens were denatured by acid, alcohol, or heating. The native and the denatured proteins were suitably dissolved, and the rotations of their solutions measured. A Hilger polarimeter (reading to 0·01°) and a mercury arc lamp were used. The solutions were made sufficiently concentrated to give readings of a reasonable magnitude, from 3° up to 6° being the average. Stronger solutions are too viscous. The concentration of protein was determined gravimetrically by coagulation, as described by Hopkins (8). It was previously found *(l.c.)* that globin became more *l*-rotary on denaturation. Serum proteins and egg albumin show a similar behaviour. The slopes of the rotatory dispersion curves of the denatured proteins are less steep than those of the native proteins. The ultra-violet absorption bands calculated therefrom have a shorter wave length in the case of the former substances than of the native proteins from which they are derived. It may be noted that Hicks and Holden (6) found that denaturation of ox globin did not alter its ultra-violet absorption curve. In some unpublished work they have found that this applies to hen ovalbumin and to some serum proteins. It its hoped to complete these experiments at a later date.

Later experiments were confined to crystalline ovalbumin. The increase in rotation was invariably observed, whether acid, alkali, or alcohol were used as the denaturing agent. A large number of unsuccessful attempts were made

EXPLANATION OF GRAPH.

Curves of Rotatory Dispersion of Proteins.

Ordinates: $\frac{1}{(a)} \times 10^8$.

Abscissae: $\lambda^2 \times 10^{-7}$.

		λ in A.U. Wavelength in A.U. of ultra-violet absorption band calculated from rotation.
A	Egg albumin	2828
B	Ox euglobulin	3605
C	Sheep euglobulin	3936
D	Egg albumin denatured by alcohol at —5°C. ..	2345
E	Ox euglobulin denatured by acid	1870
F	Egg albumin denatured by prolonged action of acid (N.HCl 5 days at 37°C.)	1225
G	Sheep euglobulin denatured by acid	2000

to prepare specimens of denatured ovalbumin of definite and constant specific rotation. While it is possible, in a general way, to prepare one of lower, or of higher, optical activity as desired, specimens prepared on different occasions by similar methods differed appreciably in this respect. Denatured proteins must be regarded at present as uncertain mixtures. For denatured egg albumin the range of variation of specific rotation of specimens prepared by different methods is considerable. They range from $[-58{\cdot}8^\circ]_{5461}$ prepared by alcohol at a low temperature to $[-99{\cdot}9^\circ]_{5461}$ after prolonged treatment with acid. Some examples will be found in Table 1. Considering the low optical activity of most amino-acids, with the exception of cystine, the magnitudes involved are rather striking. The optical activity of hydroxyprolylproline anhydride $\left[-142^\circ\right]_D^{20}$ prepared by Dakin (1) compared with those of its constituent amino-acids, hydroxy-proline $\left[-72{\cdot}5^\circ\right]_D^{20}$ and proline $\left[-86{\cdot}8^\circ\right]_D^{20}$ indicate the difficulty of any predictions in this field.

Owing to the tendency of proteins to remain associated, in spite of different degrees of solubility, it was thought at first that the varying rotations might be due to the presence of variable amounts of native protein, in spite of the fact that the denatured protein was precipitated at its iso-electric point and well washed. Incidentally, in none of the denaturation experiments recorded in this paper was any protein found to be soluble at the iso-electric point. A precipitating antiserum to pure native egg albumin was prepared from rabbits. This, while sensitive to crystalline egg albumin, gave no precipitate with any sample of denatured egg albumin, whatever its rotation. A mixture of native and denatured egg albumin in equal concentrations reacted with the serum as though only the former protein were present. Hence the variable rotations observed were apparently not due to the presence of native protein.

In addition to depending on the method of preparation, the optical activity of a specimen of denatured egg albumin depends to a considerable extent on its most recent history, as the following experiment shows. 4·8 gm. native egg albumin in 400 cubic centimetres of 0·05 N hydrochloric acid was heated on a boiling water bath for 20 minutes. The acid was neutralized, and the precipitated denatured protein washed with distilled water. A portion was kept in the solid state under water at 0°C., and the remainder dissolved in the minimum amount of sodium hydroxide. A portion of the solution was acidified and the specific rotation of the protein in each solution immediately determined. Both solutions were kept at 0°C. in closed vessels, and their rotations determined at intervals. As Table 2 shows, the alkaline solution showed a steady fall in rotation, while the acid solution showed a rise. The fall might be attributed to racemization, but the rise on the acid side is not due to the formation of any simpler breakdown products that can be detected on precipitation of the denatured protein at its iso-electric point.

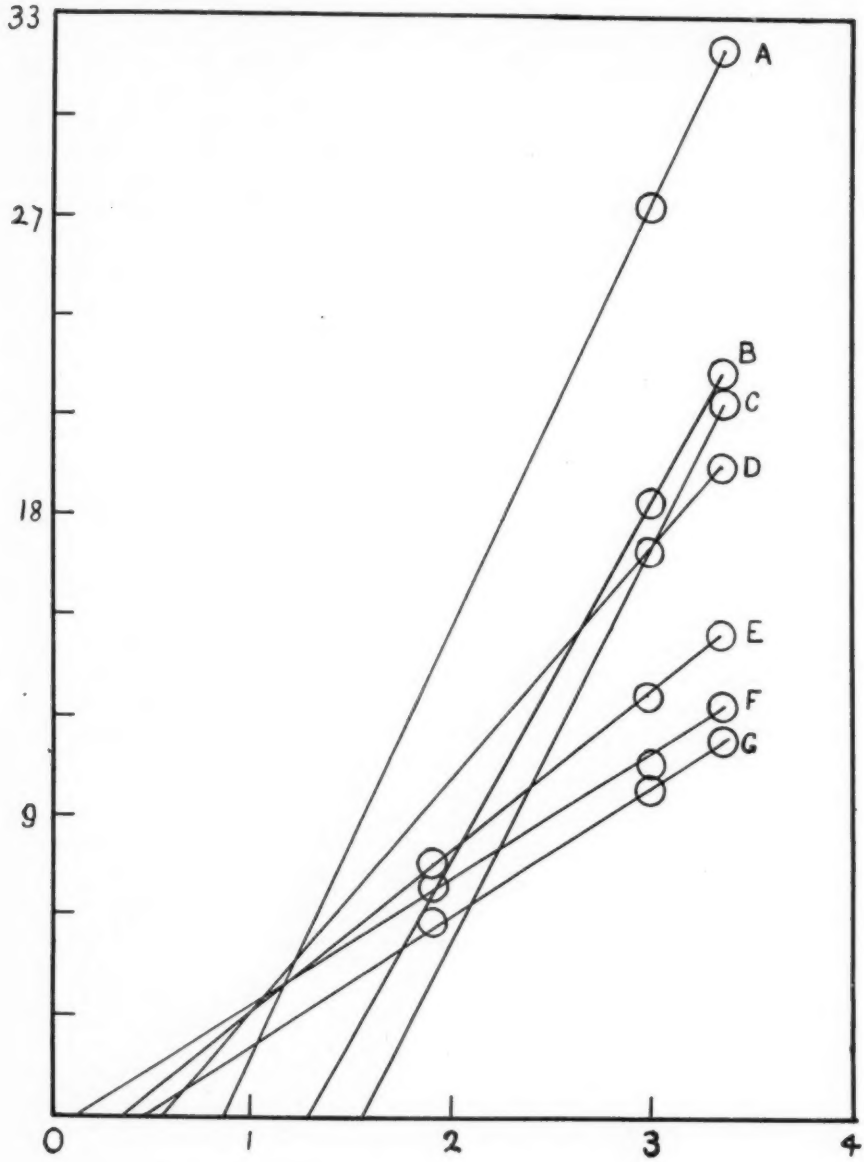

33
27
18
9
0
1
2
3
4
A
B
C
D
E
F
G

TABLE 1.

Effect of Denaturation on Specific Rotation of Proteins.

Native.				Denatured.				
Protein.	Solvent.	Specific Rotations λ in A.U.[1] 5780	5461	Denaturing Agent.	Solvent.	Specific Rotations λ in A.U. 5780	5461	4359
Ox globin	Water	—13°	—	HCl	NaOH	—72°	—	—
					HCl	—73°	—	—
Horse globin	Water	—18°	—	HCl	NaOH	—71°	—	—
					HCl	—73°	—	—
Ox euglobulin	NaCl	—44·8°	—53·9°	$\frac{N}{5}$.HCl 37°C. 18 hours	NaOH	—67·7°	—78·8°	—131·2°
Sheep euglobulin (fat free)	NaCl	—46·6°	—58·3°	HCl 37°C.	NaOH	—88·8°	—101·4°	—170·4°
Hen egg albumin	Water	—31·1°	—36·5°	Alcohol —5°C.	NaOH	—51·4°	—58·8°	—
Hen egg albumin	$(NH_4)_2SO_4$	—31·3°	—37·3°	$\frac{N}{2}$.HCl 37°C. 9 days	NaOH	—88·2°	—99·9°	—
				Alcohol 25°C.	NaOH	—55·7°	—64·8°	—
				Alcohol 25°C.	HCl	—51·6°	—60·7°	—
				N.HCl. 37°C. 5 days	NaOH	—81·2°	—94·0°	—145·0°
				Heat to 100°C. with Na_2CO_3 4·66% in 0·09% Na_2CO_3		—	—99°	—

TABLE 2.

The Effects of Recent Treatment on the Specific Rotation of Denatured Egg Albumin.

400 cubic centimetres of 1·2% native egg albumin in 0·05 N.HCl heated to 100°C. for 20 minutes. The precipitate washed with water. A portion (A) retained wet in the solid state at 0°C. The remainder (B) dissolved in dilute NaOH. A portion (C) of this solution made just sufficiently acid with HCl to redissolve the precipitate.

Date.	Solution.	Concentration.	Rotation.	Tube.	$[\alpha]_{5461}$
16.1.30	B	2·22%	—3·54	2 dm.	—79·9°
	C	2·17%	—3·59	„	—82·7°
17.1.30	B	2·22%	—3·39	„	—76·5°
	C	2·17%	—3·75	„	—86·4°
20.1.30	B	2·22%	—3·40	„	—76·7°
	C	2·17%	—3·81	„	—87·8°
28.1.30	B	2·22%	—3·33	„	—75·1°
	C	2·17%	—3·95	„	—91·0°
21.1.30	A (NaOH)	2·25%	—3·48	„	—77·4°

[1] A.U.=Ångström Unit.

Solutions in acid have higher specific rotations than those in alkali. Egg albumin denatured by alcohol had a higher specific rotation when dissolved in alkali than in acid.

TABLE 4.

Specific Rotation of Casein.

pH.	%	λA.U.	Rotation.	Tube.	Specific Rotation.
8·0	2·03	5461	—5·16°	2 dm.	—127°
6·0	2·96	5461	—3·76°	1 dm.	—127°
2·6	2·87	5461	—3·85°	1 dm.	—133°

The second example was dialysed for several days at 0°C., but no effect on the rotation was observed.

TABLE 5.

Egg Albumin Denatured with Alcohol.

Dissolved in dilute NaOH. 1·109%, 2 dm., $-1·67°_{5461}$, $[-75·3°]_{5461}$.

Some of the solid was treated with 0·5 N.HCl for 8 days at 37°C. None dissolved. After washing with water, dissolved in dilute NaOH. 3·115%, 2dm. $-5·62°_{5461}$, $[-90·2°]_{5461}$.

1·5% egg albumin in 0·5 N.HCl at 37°C. Samples removed at intervals, washed, and dissolved in dilute NaOH.

Days.	Concentration.	Tube.	Rotation (5461 A.U.).	Specific Rotation.
2	2·97%	2 dm.	—4·87°	—82·0°
3	3·01%	2 dm.	—4·91°	—82·2°
5	4·13%	1 dm.	—3·16°	—76·7°
6	4·16%	2 dm.	—7·61°	—91·5°
7	3·54%	1 dm.	—2·89°	—81·5°
9	3·53%	2 dm.	—7·07°	—99·9°

1·0% egg albumin in N.HCl.

Days.	Specific Rotation.
2	—69·4°
3	—83·0°
5	—94·0°

The Optical Activity of Crystalline Egg Albumin.

In order to familiarize themselves with the method of protein estimation employed, the writers carried out some determinations of the optical activity of crystalline hen egg albumin, using the figures of Young *(l.c.)* as a criterion of their skill. For a time they were troubled by the fact that irregular values, as high at times as $[-39°]_{5461}$, were obtained with salt-free solutions. No loss of protein could be detected, and the solutions were of suitable concentration and quite clear. The repeated crystallizations were carried out in the laboratory, the temperature of which was higher than that of an English one.

It seemed possible that these results might be attributable to the presence of small amounts of denatured protein, colloidally protected by the high concentration of native albumin, in the absence of salts. The work was repeated on some samples of egg albumin, in the preparation of which the following precautions were observed. The eggs used were all less than 24 hours old. As soon as received they were cooled in the refrigerator, in which the entire preparation was carried out. After removal of the yolks, the whites were carefully stirred with glass rods to break up the membranes, instead of being beaten to a froth. The neutral, saturated, ammonium sulphate solution was previously cooled and carefully added to the whites with constant gentle stirring. Only sufficient 10% acetic acid was used to start the crystallization, and the remainder gradually added as crystallization proceeded. In one case the additional 0·1% acid was omitted, and crystallization occurred perfectly normally. All filtration was done on Buchner funnels, and care taken to avoid superficial drying or mechanical injury of the protein. The solution was filtered after each crystallization. When these precautions were observed the values obtained for the optical activity of crystalline egg albumin in solution containing ammonium sulphate were consistent and very near to those of Young. The value after dialysis at 0°C. fell by about 1°. This change was noted by Willcock (13) and by Osborne (9). The former worker also noted that some of her preparations of egg albumin had a high optical activity. The decrease in optical activity on dialysis is apparently not due to denaturation, since in every known case it increases the rotation of the protein. It seems to indicate that crystalline egg albumin forms a compound with ammonium sulphate. Willcock's conductivity experiments would seem capable of being interpreted as suggesting that the egg albumin depresses the ionization of the salt without necessarily combining with it. It seems probable that high and irregular optical values may reasonably be explained as suggested above. It is of interest to notice that Svedberg (12) prepared crystalline egg albumin for ultra-centrifugal molecular weight estimation. He found that his specimen contained 6% of an impurity of a much higher molecular weight than that of pure albumin. The extreme liability of this protein to mechanical denaturation was emphasized by Hopkins *(l.c.)*. Whether the values given by Hopkins and by Young will need a slight revision is a question that this paper does not attempt to discuss. It seems certain that salts and hydrogen ion concentration exert modifying influences. The effect of the temperature of th polarimeter tube between 10°C. and 20°C. appears to be slight.

According to Hewitt the specific rotation of more highly purified egg albumin is $[-44{\cdot}5^\circ]_{5461}$, when all lipoid material is carefully removed by means of alcohol and ether.

Some estimations were made on the amount of such ether-soluble material present in egg albumin. The protein, in aqueous solution, was precipitated

with alcohol and ether. The solvents were filtered off through a fat-free filter into the weighed flask of a Soxhlet extraction. They were distilled off and replaced with pure ether. The dry egg albumin was placed in a fat-free thimble and thoroughly extracted. The ether was distilled off, and the flask dried at 100°C. The "blank" value, due to oxidation of the ether, was 2 milligrammes, and was subtracted from the observed increases in weights.

TABLE 3.

Weight of egg albumin.	Weight of lipoid.	Percentage of lipoid.
4·5 gm.	2·4 mgm.	0·05
2·7 gm.	1·7 mgm.	0·07
11·3 gm.	3·0 mgm.	0·03
3·0 gm.	3·0 mgm.	0·10

It will be seen that the amount present is very small. If any form of combination be assumed, the molecular weight of egg albumin would need to be far higher than that assigned to it. It would seem more reasonable to regard the "lipoid" as a minute trace of impurity. It is indeed open to question whether the apparent value is not wholly due to some effect of the protein on the ether, increasing the rate of formation of non-volatile products.

It was the experience of Sörensen *(l.c.)* that crystalline egg albumin cannot be satisfactorily precipitated with alcohol and ether at a low temperature. If this course be followed the protein so obtained is mostly converted into a form insoluble in water. Hewitt, however, used this method of purifying the egg albumin for his researches. He precipitated the protein thus after one crystallization and without dialysis. After precipitation and resolution he apparently crystallized it once more. The egg albumin so prepared had a specific rotation of $[-44\cdot5^\circ]_{5461}$. His work has been closely studied, as it was hoped to use polarimetric measurements extensively in dealing with proteins. In spite of numerous attempts to repeat his experiments on this protein it was invariably found that the proportion remaining soluble after the use of organic solvents was too small for it to be felt that the soluble protein was purified by the process to the slightest degree. The technique in this laboratory was as successful for an individual protein of serum as for the mixed proteins. It was tried on egg albumin at pH 4·8, 7·0, and 8·0, but without success. The most rigorous precautions were taken to prevent any accidental rise of temperature, but in every case only a very small percentage remained in the native state. One experiment was performed which may throw some light on the subject. Raw egg-white was precipitated by alcohol and ether at a low temperature. Here the ovo-mucoid, which is only very slowly denatured by alcohol, exerted a protective action on the albumin. A certain amount of the mixed proteins was dissolved from the dried precipitate by water. The specific rotation of the mixture was $[-94\cdot6^\circ]_{5461}$. When titrated against an antiserum to some egg albumin, carefully crystallized five times, the bulk of the

protein appeared to consist of the crystalline albumin, in spite of its high rotation. The first crystallization still leaves a fair amount of ovo-mucoid associated with the crystalline albumin. It is difficult to imagine that the Hardy technique under these circumstances yields a more highly purified protein than is obtained by repeated crystallization under appropriate conditions. It may be noted that denatured egg albumin is probably only separated from the native protein by working sufficiently on the alkaline side of their iso-electric point to enable the solution to be half saturated with ammonium sulphate without precipitating the native protein. Once the acid has been added for crystallization purposes any small amount of denatured protein formed will almost certainly remain associated with the native protein. Hence the utmost care is necessary in repeated crystallizations to guard against mechanical denaturation.

The Effect of Salts on the Solubility of Casein and of Denatured Egg Albumin near their Iso-Electric Points.

During the work on the optical activity of denatured egg albumin, solutions of this protein were required near to its iso-electric point, yet containing a fair concentration of protein. It was noticed that on the acid side very small amounts of salts sufficed to precipitate the proteins. Several solutions were accordingly prepared, in order that their behaviour towards salts might be more closely studied. Much of the research on the relations between salts and proteins suffers from the fact that the salts used alter the hydrogen ion concentration of the solution to such a degree that this effect masks any effect due to either of the ions of the salt itself. All that has been attempted in this work is to test the effect on the protein solutions of a few salts that do not possess this advantage. This consideration rules out most of the salts of the heavy metals at the concentrations of hydrogen ions of the solutions used in these experiments. For comparison with denatured egg albumin casein was used, and in certain respects these proteins show considerable similarity.

The casein was prepared from fresh separated milk by repeated precipitation and resolution. It was finally dried with alcohol, and the fat extracted with ether. The egg albumin was crystallized five times and dialyzed free from salts. It was denatured by precipitation with alcohol at 20°C., and dried with alcohol and ether. Such a preparation dissolves readily when ground with water to which a small amount of dilute sodium hydroxide solution is added. It is, however, less readily soluble than casein. A stronger solution than about 1% is prepared by precipitating the protein from a weaker solution at the iso-electric point, washing it rapidly, and redissolving either in dilute acid or alkali. In this way solutions containing 3–4% protein may be conveniently prepared. More concentrated solutions are best prepared by pres-

sure dialysis. The continuous removal of acid or alkali by this means keeps the viscosity of the solution within practical limits. The solutions of denatured egg albumin and of casein used for the experiments described in this section were dialyzed against several changes of distilled water. The effects observed on the acid side differed from those on the alkaline side of the iso-electric point. On the acid side, as dialysis proceeded, the solution became opalescent and the hydrogen ion concentration fell towards that of the iso-electric point. As this was approached the fluid became white and quite opaque. Finally the iso-electric point was reached and the protein all precipitated. This occurred regularly, in spite of the dialyzing vessels being coated with wax and the water distilled through a quartz condenser to avoid any trace of alkali. When removed from the dialyzer the solutions were quite stable in carefully cleaned glass vessels. The precipitation of these two proteins by membrane hydrolysis contrasts with the behaviour of ox serum euglobulin, which, dissolved in acid, was not precipitable by dialysis (Hardy, 3).

On the alkaline side of the iso-electric point it was found impossible to remove the alkali completely by prolonged dialysis. It was also found impossible to prepare a stable solution as near to the iso-electric point as on the acid side. A very opalescent solution could be obtained temporarily by the addition of dilute acid to about pH 5·1 to a dialyzed solution of either protein in dilute alkali, but part of the protein was precipitated on standing.

The salt precipitation experiments were conducted in the following manner. 2 cubic centimetre samples of the protein solution, containing about 0·4–0·5% protein, were measured into carefully cleaned test-tubes. The solution of the salt was added slowly from a micro-burette until the protein was precipitated and the supernatant fluid clear. The salt solutions were all neutral. Salts of feeble acids or bases were avoided, owing to their effects on the hydrogen ion concentration of the liquid. The two proteins resemble each other in their general behaviour towards the electrolytes used. As can be seen from the tables, the amounts required for precipitation near the iso-electric point are very small. On the alkaline side salts of monovalent kations had no effect in moderate concentration. It is of interest to notice that potassium nitrate was a more effective precipitant than potassium chloride, although both acids are of comparable "strengths." The precipitates were readily soluble in dilute nitric acid.

The behaviour of these proteins towards mercuric chloride is of interest. On the acid side of the iso-electric points it has no effect on the hydrogen ion concentrations of the solutions or on the precipitating action of sodium chloride. It has no precipitating power of its own, even if a saturated solution be added to the protein solution. On the alkaline side of the iso-electric points of the proteins it precipitates in the zone between the iso-electric point and about pH 8. If the solution be more alkaline no precipitate is obtained. The

mercury-protein precipitates are soluble in dilute acid, alkali, or sodium chloride solution. If the solution in sodium chloride be dialyzed the mercuric chloride is removed at the same time as the sodium chloride, and a solution of the protein on the alkaline side of its iso-electric point remains in the dialyzer. Denatured horse globin, which has an iso-electric point at pH 8·1, shows an analogous behaviour. This protein, however, is sensitive to small amounts of salts through a wide range, which masks this effect somewhat. Owing to this peculiarity the mercuric chloride precipitate of this protein is not soluble in sodium chloride solution. This behaviour is comprehensible when it is seen that the degree of ionization of mercuric chloride (about 2–2·5%) is such that it cannot provide sufficient chloride ion for precipitation. On the alkaline side the amount required is so much less than that of other divalent chlorides that the effect must be regarded as specific. A smaller volume of a solution of mercuric chloride than of an equivalent solution of hydrochloric acid is required for precipitation.

TABLE 6.

Denatured Egg Albumin in HCl. 0·8% pH 4·77.

5 cubic centimetres titrated with 0·0004 N. NaOH.
No precipitate with 1·24 cubic centimetres.
Precipitation complete with 1·28 cubic centimetres.
From beginning to end of precipitation=4×10^{-7} equivalents per gram.

Salt.	Concentration.	cc. for complete precipitation of 2 cc. protein solution.
NaCl	0·02 N.	0·55
KCl	0·02 N.	0·54
NH_4Cl	0·02 N.	0·51
KNO_3	0·02 N.	0·29
$KClO_3$	0·02 N.	0·33
$CaCl_2$	0·02 N.	0·54
$SrCl_2$	0·02 N.	0·55
$BaCl_2$	0·02 N.	0·62
Na_2SO_4	0·004N.	0·44
K_2SO_4	0·004N.	0·42
$(NH_4)_2SO_4$	0·004N.	0·40

Not precipitated by $HgCl_2$.

Denatured Egg Albumin in NaOH. pH 6·1. 0·46%.

Salt.	Concentration.	cc. for precipitation of protein in 2 cc. solution.
$CaCl_2$	0·02 N.	0·78
$SrCl_2$	0·02 N.	0·75
$BaCl_2$	0·02 N.	0·78
$HgCl_2$	0·005N.	0·16

The mercuric precipitate is soluble in dilute acetic acid, sodium carbonate, or sodium chloride.

Salts with monovalent kations did not precipitate the protein.

TABLE 7.

Casein in HCl. 0·77%. pH 4·26.

Salt.	Concentration.	cc. for precipitation of protein in 2 cc. solution.
NaCl	0·02 N.	1·22
NH_4Cl	0·02 N.	1·53
KCl	0·02 N.	1·28
KNO_3	0·02 N.	0·76
$KClO_3$	0·02 N.	0·86
$CaCl_2$	0·02 N.	1·51
$SrCl_2$	0·02 N.	1·49
$BaCl_2$	0·02 N.	1·47
Na_2SO_4	0·004N.	0·96
K_2SO_4	0·004N.	1·07
$(NH_4)_2SO_4$	0·004N.	0·96

5 cc.=0·69 cc. of 0·004N. NaOH.
1 gm. casein=$7·4\times10^{-5}$ equivalent.
Another solution 0·45% pH 4·4.
2 cc.=0·56 cc. 0·001 N. NaOH.
1 gm.=$6·3\times10^{-5}$ equivalents.
$HgCl_2$ did not precipitate the casein in HCl.

Casein in NaOH. 0·46%. pH 6·4.

Salt.	Concentration.	cc. for precipitation of protein in 2 cc. solution.
$CaCl_2$	0·02 N.	0·37
$SrCl_2$	0·02 N.	0·40
$BaCl_2$	0·02 N.	0·39
$HgCl_2$	0·005N.	0·18 ($9·8\times10^{-5}$ equivalents per gram)

The mercuric precipitate dissolved in dilute NaOH, HCl, or NaCl.

DISCUSSION.

The problems connected with the denaturation of proteins beset every worker in this field, from the physical chemist interested in their physical constants to the biological worker who desires uniform material for serological or physiological investigations. It would seem by no means easy to prepare even a single crystalline protein, such as ovalbumin, entirely free from denatured products. The latter, as far as present knowledge goes, consist of complex mixtures. Though insoluble at their iso-electric points, they are readily soluble in dilute acid or alkali. The amount necessary for their solution is extremely minute, smaller than that required to dissolve an equal weight of casein. The state of these substances in solution seems still uncertain. On both sides of the iso-electric point both denatured egg albumin and casein, when held in solution by very small amounts of acid or alkali, show marked opalescence. Both substances have considerable "protective" powers. Proteins in solution show great tendency to associate in spite of differing solubilities. Although under these circumstances the protein all moves in an electric field,

the whole is not necessarily ionic, i.e., combined with acid or alkali. A portion, perhaps a considerable proportion, may be non-ionic, colloidally protected by the ionized part. Hardy suggested this explanation for serum eu-globulin in an analogous state. Tho whole protein becomes ionic, probably, when the solution becomes quite clear. From the figures obtained by titrating these opalescent solutions of protein with alkali the equivalent (or minimal molecular weight) of denatured egg albumin would be $2{\cdot}5 \times 10^6$, and a correlation of the equivalents for casein dissolved in acid and in alkali would lead to an enormous molecular weight.

It would appear questionable whether such figures are capable of carrying the superstructure of calculation that has been based thereon. The preparation of uniform denatured proteins with accurately defined characteristics appears to be not yet practicable. They can be freed from native protein by repeated solution and reprecipitation, and by a prolonged use of the denaturing agent. The preparation of a native protein free from denatured protein is seen to be a somewhat difficult task except for proteins of a few limited classes. One of the workers (H.F.H.) has prepared ox globin which gave no haemochromogen test when ferrous tartrate and sodium carbonate were used. Egg albumin may have any denatured protein removed by half-saturation with ammonium sulphate in neutral solution. Once the solution has been adjusted to pH 4·8 for crystallization no further quantitative removal is possible at that hydrogen ion concentration. A certain amount appears to be "protected" by the native albumin. In consequence the utmost care is advisable to guard against the small amount of mechanical denaturation liable to occur during several recrystallizations. It is doubtful whether, if the filtration and washing are carefully performed, any benefit is derived from increasing the number beyond Hopkin's recommendation of two. The polarimetric method is suggested as the best for detecting relatively small amounts of denatured protein in a specimen of native protein, provided that the salt and hydrogen ion concentrations are specified.

The effect of small concentrations of salts on the solubilities of denatured egg albumin and of casein shows their great effect in apparently broadening the iso-electric zone, particularly on the acid side. It is of interest to note that casein behaves in this respect similarly to a denatured protein, and that in the case of egg albumin denaturation changes it from one which does not exhibit the behaviour of a typical colloid, as described by Loeb, to one that does.

SUMMARY.

The specific rotation of crystalline hen ovalbumin in ammonium sulphate solution at pH 4·8 is about $[-37{\cdot}3^\circ]_{5461}$. On dialysis the value falls to $[-36{\cdot}5^\circ]_{5461}$. The value of Hewitt $[-44{\cdot}5^\circ]_{5461}$ cannot be confirmed. An explanation is suggested of his figure.

On denaturation of proteins their specific rotations increase. The amount of the increase is variable, depending on the method of denaturation and subsequent history of the denatured protein.

Casein has a slightly higher specific rotation in acid than in alkaline solution.

The effects of salts on solutions of casein and of denatured egg albumin are compared.

The peculiar effect of mercuric chloride is discussed.

REFERENCES.

1. Dakin, H. D.: Journ. Biol. Chem., 44 (1920), p. 499.
2. Gamgee, A. and Hill, A.: Beit. z. chem. Physiol. u. Pathol., 4 (1904), p. 1.
3. Hardy, W. B.: Journ. Physiol., 33 (1906), p. 330.
4. Hardy, W. B. and Gardiner, Mrs. S.: *Ibid.*, 40 (1910), p. lxviii.
5. Hewitt, L. F.: Biochem. Journ., 21 (1927), p. 216.
6. Hicks, C. S. and Holden, H. F.: Aust. Journ. Exper. Biol. and Med. Sci., 6 (1929), p. 175.
7. Holden, H. F. and Freeman, M.: Aust. Journ. Exper. Biol. and Med. Sci., 5 (1928), p. 213.
8. Hopkins, F. G.: Journ. Physiol., 25 (1900), p. 306.
9. Osborne, T. B.: Journ. Am. Chem. Soc., 21 (1900), p. 477.
10. Schulz, F.: Zeits. für physiol. Chemie., 24 (1898), p. 449.
11. Sörensen, S. P. L.: Journ. Chem. Soc., (1926), p. 3007.
12. Svedberg, T. and Nicholls, J. B.: Journ. Am. Chem. Soc., 48 (1926), p. 3081.
13. Willcock, E. G.: Journ. Physiol., 37 (1908), p. 27.
14. Young, E. G.: Proc. Roy. Soc. London, 93B (1922), p. 15.

A PHYSICAL DIFFERENCE AMONGST BACTERIOPHAGES

by

F. M. BURNET

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 16th May, 1930.)

There are well-defined functional differences amongst bacteriophages which persist indefinitely on passage. A suitably chosen set of phages propagated on a single substrate organism will show the same general characteristics after each successive lysis. The most useful of such functional characteristics for the purposes of classification are the average size of the plaques produced on agar under standard conditions and the type of resistant culture obtained after lysis. In the course of work on salmonella and dysentery bacteriophages a large number of pure phages of different functional types has been accumulated. The differences amongst these phages are often very striking. The plaque size, for instance, may vary from about 0·1 millimetre to over a centimetre in diameter on 1·5% agar. Such differences are quite definitely to be related to the character of the phage particles used, since they are manifested when all the other factors involved are identical. Attempts have therefore been made to determine whether any differences in the physical qualities of the phages can be correlated with these functional differences.

The present paper is concerned with a well-marked physical difference amongst phages, which, although it cannot be correlated with functional type, seems to demand close consideration when any work on the purely physical behaviour of bacteriophages is being undertaken. All the phages acting on the ordinarily used intestinal bacilli are moderately resistant to environmental influences when preserved in the usual form of a filtrate from a lysed broth culture, but when diluted a hundredfold or more in a non-nutrient fluid, such as normal saline or phosphate solution, a fairly sharp differentiation into resistant and non-resistant phages can be made.

This differentiation first became apparent when the power of phages to ascend strips of filter paper was being studied according to the method of Bedson and Bland (2). Certain phages appeared to ascend in undiminished concentration as far as the paper was wet by the fluid, while others rose only about half as far as the fluid in which they were diluted. This difference was

found to be correlated with the heat resistance of the same phages when diluted in phosphate. The phages which failed to rise well in filter paper were inactivated completely, or almost completely, when heated to 55°C. for half an hour. Those that rose in undiminished amount were only slightly weakened by this amount of heating.

EXPERIMENTAL.

The series of phages used was chosen to present as wide a range of functional type as possible. They have all been described previously along with the methods used for their classification (Burnet 5, Burnet and McKie 6). In the tabulation below (Table 1), the groups C, D, E, and N represent the type as judged by the nature of the resistant cultures produced, the phages are prefixed S or D according to whether they have been used with salmonellas or dysentery bacilli, and the average plaque size is indicated. Large, medium, and small correspond to plaques >2·5 millimetres, 1–2·5 millimetres and <1 millimetre in diameter on 1·5% agar, respectively.

TABLE 1.

Bacteriophages Used.

Plaque Size.	Type.			
	C	D	E	N
Large	D 5	D 3, S 13	D 21	D 13, D 20
Medium	S 18	S 25	D 6	
Small	S 8	D 4	D 33	D 12

Strips of Whatman No. 1 filter paper, 15 x 1 cm., were sterilized in the autoclave and suspended with about 3 mm. dipping into the phage dilution, which was contained in watch glasses. The experiments were carried out in a dust-free chamber kept saturated with water vapour. The fluid rose approximately 10 cm. in an hour, and it was found convenient to leave the strips about 75–80 minutes, when the level attained was about 12 cm. above the liquid surface. The strips were then removed, and portions, subsequently measured, were cut off with sterile scissors and placed in 5 cc. of sterile saline. These tubes were shaken at intervals, and after half an hour the fluid titrated for its phage content on agar spread with a suitable culture. In order to give complete controls practically all experiments were carried out with mixtures of two phages in phosphate. The mixtures were chosen so that the plaques due to each phage could be readily recognized, e.g., phages D 20 and D 33, both active on a Flexner Y smooth strain, could be differentiated simply by the difference in size and appearance of their plaques, or in the case of D6 and

D13, the former was titrated on a smooth Flexner strain, the latter on a rough strain, these being "pure smooth" and "pure rough" phage types respectively. Details will be given of a typical experiment carried out with the dysentery phages 20 and 33. The results with other phages will be summarized.

Experiment 1. Phages D 20 and D 33 were added to 5 cc. of sodium phosphate solution (3%) at pH. 7·4. The amounts (5 drops of phage 33 and 2 drops of a 1 : 10 dilution of phage 20) were adjusted to give similar numbers of particles of each phage. This solution was used for a filter paper rise experiment according to the method described. Titrations were carried out in duplicate at two dilutions of the mixture before the experiment and of the extracts from segments of the paper at the average heights shown above the fluid. A smooth Y strain was used, and counts of both phages made from the same plates. The plaques of each type were very distinct in their appearance, and there was never any doubt as to the type of a given plaque.

TABLE 2.

Average height of segment.	Plaques obtained. Phage 20 Undil.		Phage 20 1:5		Phage 33 Undil.		Phage 33 1:5		Corresponding to phage particles per sq. cm. (average). Phage 20	Phage 33
10·5 cm.	0	0	0	0	161	143	19	19	Nil	31900
7·6 „	5	8	2	0	150	130	32	33	1210	28100
0·9 „	+	+	18	13	±	±	31	24	17200	29600
Control	+	+	57	51	+	+	116	97	(15200)	(29900)

The actual plaque counts are shown + and ±, indicating numerous plaques too closely packed to be readily counted. In the final column the corresponding numbers of particles calculated to be present in 1 sq. cm. of filter paper are given. The control counts are arranged to indicate how many particles of each phage would be present in a sq. cm. of paper if the mixture were uniformly distributed, the average of the counts actually obtained with phage 33 being taken as the standard for that phage.

Several repetitions gave similar figures. Phage 20 never rose more than 8 cm. above the fluid level in any experiment of this type, while 33 was always detectable in the uppermost level wet by the fluid, usually in undiminished amount, occasionally rather less than at the lower levels.

The same experimental procedure was applied to the other phages shown in Table 1, with the results tabulated below. The figures shown represent the number of particles per sq. cm. of filter paper at the average height shown. In each case the results shown for upper and lower segments were obtained in the one experiment.

TABLE 3.

Extent of Rise in Filter Paper Strips.

Phages.	Average height.	Particles.	Percentage of particles in upper segment.
D 3	10·2 cm.	510	0·3%
	1·4 cm.	173000	
D 4	10·7 cm.	2880	35%
	1·4 cm.	8320	
D 5	10·4 cm.	510	0·24%
	1·6 cm.	228000	
D 6	11·1 cm.	0	<1%
	2·0 cm.	12600	
D 12	10·6 cm.	1350	25%
	1·3 cm.	5460	
D 13	11·1 cm.	11100	>100%
	2·0 cm.	9200	
D 20	10·5 cm.	0	<1%
	0·9 cm.	17200	
D 21	10·6 cm.	1850	39%
	1·3 cm.	4680	
D 33	10·5 cm.	31900	>100%
	0·9 cm.	29600	
S 8	10·2 cm.	0	<1%
	1·4 cm.	53600	
S 13	11·3 cm.	1020	100%
	1·4 cm.	1020	
S 18	10·4 cm.	27400	73%
	2·8 cm.	37800	
S 25	10·5 cm.	0	<1%
	1·4 cm.	17800	

There is a fairly sharp differentiation into two types. Phages D 4, D 12, D 13, D 21, D 33, S 13, and S 18 rise readily, the upper segment showing from 25–100% of the particles found in the lowest sq. cm. of the strip. The others show less than 1% in the higher segment.

The same group of phages was tested for heat stability in phosphate solution according to the following technique: 0·2 cc. of the stock phage filtrate was added by a capillary pipette to 5 cc. of phosphate solution. From this a fairly thick capillary was filled and sealed at both ends. The capillaries were heated wholly immersed in a waterbath at 55°C. for 30 minutes. A drop of the heated phage solution was then blown directly on to a suitably spread plate, and another into 5 cc. of saline, which was also titrated. On this method the size of the drop from the capillary probably varied from about 0·02–0·03 cc., so that only considerable differences can be judged as significant. The table shows the plaque counts obtained from a single standard drop (0·03 cc.) of the unheated and heated phosphate solutions (average of 2 counts in each case).

TABLE 4.

Effect of Heating on Phages Diluted in Phosphate.

Phage.	Unheated.	After 55° for 30 minutes.	% "surviving."
D 3	400	0	<0·25
D 4	2,300	1,150	50
D 5	40,000	2	0·005
D 6	522	0·5	0·1
D 12	160	12	7·5
D 13	3,760	30	0·9
D 20	132,000	13	0·01
D 21	4,620	56	1·2
D 33	7,260	276	3·8
S 8	5,190	4·5	0·09
S 13	1,130	414	32
S 18	13,800	118	0·85
S 25	1,480	0	<0·01

If we arrange this series in order of increasing heat stability we find the following: D 3, D 5, D 6, D 20, S 8, S 25 : S 18, D 13, D 21, D 33, D 12, S 13, D 4. It is evident that the first six very labile phages are also the ones which fail to ascend freely in filter paper.

In order to see whether this difference in heat resistance amongst phages persisted on passage, six representative phages were reisolated from single plaques, and grown (a) anaerobically in meat medium tubes sealed with solid paraffin, and (b) aerobically in slanted broth tubes according to Nanavutty's method (12). The heat resistance of the phages so prepared was tested as previously, and the results tabulated as percentage of "survivors" are given in Table 5.

TABLE 5.

Heat resistance of phages grown in different ways: showing percentage of particles capable of producing plaques after 30 minutes at 55° C.

Phage.	Stock.	Anaerobic.	Hyperaerobic.
D 4	50%	80%	50%
D 5	0·005%	0·02%	0·012%
D 6	0·1%	0·1%	0·01%
D 20	0·01%	0·0004%	0·005%
D 33	3·8%	6·8%	3%
D 13	0·9%	<0·3%	0·03%

With the exception of phage D 13, and to a lesser degree phage D 20, the characteristic degree of resistance of each phage is well shown to persist despite different modes of growth. The anomalous behaviour of phage D 13 was found to depend on the age of the filtrate. In this case the stock filtrate was some weeks old, while the other two were tested on the day they were filtered. The experiment was repeated later, using the same "hyperaerobic" filtrate, now three weeks old, and a freshly grown filtrate of the phage. The three weeks old phage showed 0·37% of "survivors," the new filtrate 0·029%. d'Herelle has described this increased resistance to heat with the ageing of

certain phages. Like so many other characteristics of phages, it seems to be much more marked in certain strains than in others.

The reason for the correlation between relative heat stability and readiness to ascend filter paper is not obvious. The apparent failure of certain phages to rise beyond a certain level in filter paper may be due either to a true inability of the particles to ascend or to an inactivation of particles that have ascended normally. The most likely factor involved is a firm adsorption of the sensitive phage particles to the fibres of the paper. This would act in both ways, by progressively diminishing the number of free rising particles as the height increases, and by holding such particles as do rise so that they cannot be estimated by the method used. Apart from adsorption one must consider the possibility of other causes of "spontaneous" inactivation being more effective in the upper levels. The results of some experiments on adsorption by filter paper may be given.

Experiment 2. Phages D 20 and D 33 were suitably diluted in phosphate solution, to give a mixture of which 1 drop contained a conveniently counted number of particles of each phage. The mixture was titrated and divided between two tubes, to one of which 10 sq. cm. of filter paper was added. Both tubes were left in the dark and shaken at intervals. After the times shown both tubes were titrated. Two similar experiments with different times of exposure are tabulated.

TABLE 6.

	Expt. A (70 mins.)		Expt. B (140 mins.)	
	Phage 20.	Phage 33.	Phage 20.	Phage 33.
Original mixture	64	15	250	235
Control after time shown	33	8	111	208
With filter paper after same period	22	15	60	193

In this experiment phage 33 is relatively unaffected by exposure with or without filter paper. Phage 20 is very considerably reduced by simple exposure and significantly further if filter paper is added.

Another sensitive phage, D 6, showed a slightly different behaviour. When tested in a mixture with phage 13, the results shown in Table 7 were obtained.

TABLE 7.

	Phage 6.	Phage 13.
Original mixture	48	98
Control at 70 minutes	52	93
With filter paper at 70 minutes ..	23	70

Here there is no diminution on simple exposure, but a greater adsorption of phage 6 than phage 13 when filter paper is added.

Experiment 3. A suitable mixture of phages 6 and 13 in phosphate solution was titrated, and 0·025 cc. was delivered from a capillary pipette on

to the centre of a strip of sterile filter paper. After 10 minutes this amount had moistened an area of 3·9 sq. cm., the lower margin of which was 4·1 cm. from the end of the strip. Immediately after the mixture was applied the strip was suspended in a moist chamber dipping into phosphate solution without phage. The rising moist area fused with the central one after 8 minutes, and at the end of an hour the total moist length was 9·35 cm., 1·4 cm. above the original upper level of the central moist area. The strip was removed, and divided into four pieces of the lengths shown, so that the highest segment (1) and the lowest (4) both lay outside the region on which the mixed phage was delivered. The four pieces were placed separately in 5 cc. tubes of saline and the contents titrated after half an hour.

TABLE 8.

Height of portions tested.	Plaque counts of Phage 6.	Phage 13.
(1) 7·95—9·35 cm.	0	33
(2) 5·65—7·95 cm.	0	11
(3) 3·1 —5·65 cm.	1	1
(4) 0 —3·1 cm.	0	0

The amount of mixture added to the strip gave when correspondingly diluted 63·5 (average) plaques of phage 6 and 65 of phage 13, so that while over 70% of phage 13 was recovered only a trace of 6 was detectable. In addition it willl be noticed that phage 13 has been freely transported upwards by the capillary flow, while the only positive evidence of phage 6 was obtained from the lower of the two segments on which the mixture had been deposited.

From these experiments it seems clear that the heat sensitive phages 6 and 20 differ from phages 13 and 33 as regards their behaviour toward filter paper in being more actively adsorbed to the fibres of the paper, and so hindered from rising passively with the fluid in which their particles are suspended. It is conceivable that the sensitive phage particles carry a smaller negative charge than the more resistant types, or they may even be positively charged,[1] but no direct experiments along these lines have yet been carried out.

When phages are diluted in nutrient broth instead of phosphate solution the differences amongst them are absent or much less distinct. Phages D 4, D 6, D 20, and D 33 were heated diluted 1 : 250 in broth for 1 hour at 55°C. Phage D 20 showed a just detectable decrease to about 80% of its original value; the others showed no significant change. In filter paper experiments the type of phage used shows a definite influence when broth is the diluting medium, though the differentiation is much less striking than in phosphate solution. In one experiment with phages D 4 and D 20 diluted in nutrient broth the following results were obtained:

[1] There are conflicting reports on the electric charge of phage particles. E.g., Koch states that the phage Landa g. (Bail) carries a slight + charge, although the great majority of authors, d'Herelle, Todd, Krueger, etc., are agreed that most are negatively charged.

TABLE 9.

Average height.	Phage 4.	Phage 20.
10·9 cm.	12,200	9,350
1·6 cm.	10,540	156,000

Phage 20 rises more readily than in phosphate, but there is still a sharp distinction between the two types.

DISCUSSION.

The difference in the behaviour of phage in the form of a broth filtrate and when diluted in some non-nutrient medium has been well recognized. The increased sensitiveness to various environmental influences has been described by Krueger and Tamada (10), Beard (1), Nanavutty (11), and others. Another effect of broth constituents on the behaviour of phage has been described by Bronfenbrenner (3), and Bronfenbrenner and Hetler (4). They found that phage samples purified from broth constituents could be passed through certain collodion membranes with considerable loss of activity. The filtrates after such passage, however, could be again passed through similar filters without any further loss of activity. Apparently the smaller particles of the phage sample had passed through the membrane, while the larger ones were retained. If a membrane which had held back part of a phage preparation were now washed with broth a further amount of phage could pass through; the broth seemed to have altered the state of aggregation of the phage particles so as to render them smaller or at least more diffusible.

The chief object of the present paper is to stress the point frequently made by d'Herelle that the wide individuality of phages must be considered in any investigations into their physico-chemical aspects. It has been shown that the difference produced by the change of suspending medium from broth to non-nutrient phosphate varies enormously from phage to phage, but is constant for a given strain of phage. The titre of phage D 4 is hardly affected by heating to 55°C. in phosphate, while phage D 20 is reduced to 0·01% or less in half an hour. A glance at Table 1, where the heat sensitive phages are underlined, shows that there is no correlation between the functional attributes of a phage, as judged from the size of plaque produced and its specific type, with its heat stability in phosphate solution or its power to ascend in filter paper. It has been stated that small plaque phages are more resistant than large plaque phages (Watanabe, 14). This is often true (cf. phages D 4 and D 20), but it is not universal, since, for example, phage S 13, which produces plaques nearly a centimetre in diameter, is much more resistant than the small plaque phage S 8.

There is no evidence available that will allow a suggestion as to the intimate significance of the differences between phages in this respect, just as up to the present there have been no suggestions as to the physical basis of the differences in plaque size that characterize different phages. The only generalization apparent is the purely biological one that any phage reproduces itself

true to type in all of the characteristics that can be used as distinguishing qualities.

Finally, it would appear obvious that any physical investigations of bacteriophage, particularly measurements of size, electric charge and adsorbability, must be applied to a wide range of bacteriophages if any generally valid conclusions are to be drawn. It seems specially important that extremes of the range of variability in regard to heat stability in saline or phosphate (e.g., D 4 and D 20 above) should be included in any such investigation.

SUMMARY.

1. From a study of the height to which bacteriophages acting on bacilli of the salmonella-dysentery groups will ascend in strips of filter paper when diluted in phosphate solution they can be differentiated into two fairly sharply defined groups.

2. Phages which ascend filter paper readily are relatively resistant to heating in phosphate solution, while those that rise only a limited distance are much more rapidly inactivated by heat.

3. The characteristic heat resistance is constant for a given phage and independent of the method used for growing the phage.

4. When phages are diluted in nutrient broth, instead of a non-nutrient saline solution, these differences are slight or absent.

5. The differences observed are probably related to definite physical differences in the phage particles concerned, and the existence of the two types must be borne in mind in any studies on the physical characteristics of the phage particle.

REFERENCES.

1. Beard, P. J.: Proc. Soc. Exper. Biol. and Med., 26 (1929), p. 880.
2. Bedson, S. P. and Band, J. O. W.: Brit. Journ. Exper. Path., 10 (1929), p. 67.
3. Bronfenbrenner, J. J.: Journ. Exper. Med., 45 (1927), p. 873.
4. Bronfenbrenner, J. J. and Hetler, D. M.: Proc. Soc. Exper. Biol. and Med., 27 (1930), p. 263.
5. Burnet, F. M.: Journ. Path. and Bact., 32 (1929), p. 15.
6. Burnet, F. M. and McKie, M.: Journ. Path. and Bact., (1930) (in the press).
7. d'Herelle, F.: "The Bacteriophage and its Behaviour" (Baltimore, 1926).
8. Koch, K.: Centr. f. Bakt., etc., 1 Orig., 99 (1926), p. 209.
9. Krueger, A. P., Ritter, R. C. and Smith, S. P.: Journ. Exper. Med., 50 (1929), p. 739.
10. Krueger, A. P. and Tamada, H.: Journ. Gen. Physiol., 13 (1929), p. 145.
11. Nanavutty, S. H.: Journ. Path. and Bact., 33 (1930), p. 203.
12. Nanavutty, S. H.: Brit. Journ. Exper. Path., 11 (1930), p. 7.
13. Todd, C.: Brit. Journ. Exper. Path., 8 (1927), p. 368.
14. Watanabe, T.: Arch. f. Hyg., 92 (1924), p. 1.

FURTHER OBSERVATIONS ON CERTAIN ALLEGED EFFECTS OF SYMPATHETIC NERVES ON SKELETAL MUSCLE

by

A. E. COATES AND O. W. TIEGS

(From the Departments of Anatomy and Zoology, University of Melbourne).

(Submitted for publication 2nd April, 1930.)

Since the publication of our last paper (1), describing a series of negative attempts to confirm the work of various observers who have reported effects of sympathetic nerves upon postural tone of skeletal muscle, there have appeared several statements in recent literature in which positive results are claimed.

G. and F. Popa (5) find that if, in the pigeon, from one to five sympathetic ganglia are severed from their attachments to the brachial nerves (C. 11–15) and then removed, there occurs a droop in the wing, thus confirming the conclusion of Hunter (3) that the sympathetic nerves subserve plastic tone in the wing musculature. Hunter based his experiment on Langley's statement that the first thoracic nerve is the first by which preganglionic fibres emerge from the cord; so that severing the sympathetic chain immediately in front of this root should suffice to abolish all tone due to sympathetic action. We ourselves failed to obtain Hunter's effect. Meanwhile Popa states that "Langley's account of the sympathetic in birds ignores the presence of white rami communicantes (preganglionic fibres) above the 14th spinal nerves," thus depriving the bird's wing of the special anatomical peculiarity upon which Hunter relied for his demonstration. Hunter's own observation (if Popa's statement be correct) must therefore clearly be wrong. But it is only necessary to make a dissection of the pigeon's brachial plexus to realize that a droop in the wing following removal of the attached sympathetic ganglia cannot afford valid evidence to prove that sympathetic nerves play any direct part in maintaining muscular tone, for the ganglia are so firmly attached to the spinal nerves that it is quite impossible to remove them without doing injury to the adjacent spinal nerve fibres. Thus in figure 1 is shown a transverse section of the cervical cord of a pigeon at the level of C. 13. The bony vertebra has been carefully broken away without injuring the nerve roots; d.r. is the dorsal root, s.g. the sensory ganglion, sy.g. the sympathetic ganglion. The sympathetic ganglion is so intimately associated with the main nerve trunk that its removal must inevitably produce injury to the latter. When it is remembered that there are five such ganglia, that they are about the size of a pin's head, and that they

are comparatively inaccessible, it seems clear that injury to the brachial nerves, with consequent droop in the wing, could not possibly be avoided.

According to Gaissinsky and Lewantowsky (2), muscle from the dog's limb, when deprived of its sympathetic supply, exhibits histological degeneration. There is a "progressive loss of transverse and longitudinal striation, hyalinization, and an increase in connective tissue. . . . . The histological changes are degenerative, and do not seem to be dependent on changes in the vascular supply." How trophic influences due to interference with the blood supply have been eliminated the authors do not state. Meanwhile we have not been able to confirm the histological observation. In a dog the abdominal sympathetic chain on one side was removed as far as the first sacral ganglion, so that all preganglionic connections to the foot were destroyed. A year later the animal was killed and the interosseus muscles injected with 10% formalin through the femoral artery. After several weeks in formalin sections were cut from frozen material, and mounted either in glycerine unstained, or stained by Van Gieson's stain. Similarly treated material from the unoperated foot was used as a control. We could detect no difference between the two muscles. It is true that there are found in the operated muscle regions where the striations are indistinct, but such areas are also to be found in the preparations from the unoperated side. Nevertheless, in the preparations as a whole no significant difference between the two sides, whether in distinctness of striation or of longitudinal fibrillation, could be detected (see figures 2 and 3). Data of a quantitative nature may be obtained by comparing the diameters of muscle fibres from the operated and unoperated sides. The average thickness of normal fibres gave a value of $16\frac{1}{2}\mu$ (40 measurements); a similar number of measurements from the operated side gave a value of 17μ. The difference is obviously of no significance.

In a recent paper Royle (6) describes experiments purporting to show that stimulation of sympathetic nerve endings by drugs causes increased tonus of the skeletal musculature in the goat. If the abdominal sympathetic chain be removed, and time allowed for the sympathetic nerve terminals to degenerate, then on injecting adrenaline or ephedrine into the jugular vein all limbs except the one deprived of its sympathetic nerves become rigid (occasionally the homolateral forelimb may also remain flaccid); but if the two procedures be performed on the same day, it is stated that no such effect is obtained, since the nerve terminals have not had time to degenerate. These experiments were planned on the erroneous assumption that these "sympathomimetic" drugs stimulate endings of nerves; but it has been shown (Elliot, Lewandowsky, Langley) that they act upon the peripheral structures (muscle fibres) themselves, and not upon the nerve endings, for their action (at any rate, so far as plain muscle is concerned) persists even when all sympathetic nerves have been thrown into degeneration.

We have repeated Royle's experiment, but have failed to obtain the effect

described. In a goat the left abdominal sympathetic trunk was removed and three months allowed for degeneration. The test was then carried out as follows: Under light ether anaesthesia the jugular vein was exposed. Anaesthesia was continued till all the limbs became flaccid, but with the knee jerk and eye reflexes persisting. Into the jugular vein 0·2 gramme ephedrine hydrochloride was injected and anaesthesia discontinued. With knee jerks apparently normal the limbs remained flexed, and no attempt was made at extension. No difference could be detected between operated and unoperated limb, whether in their attitude, firmness of muscles, or character of knee jerk. The only noticeable effect of the drug was the greatly increased rate of heart beat and of respiration. After about three minutes, when the latter effect was subsiding, another injection of 0·2 gramme ephedrine was given. The result was exactly the same, and the limbs remained flaccid though the tendon jerk was active. About three minutes later a third similar injection was given, again with similar results. But after about five minutes the stiffening of the limb described by Royle suddenly appeared. The head was strongly retracted, and as Royle described, the effect lasted a few minutes only. But our observations differ from those of Royle in this respect, namely, that all limbs became rigid, and definitely no difference could be detected between the behaviour of the operated leg and its fellow. There was certainly not the slightest indication of one leg remaining flaccid while the other became rigid. The general appearance of the animal was one of "stretching" itself.

In this animal, and in the others which we employed in our previous experiments (1), no consistent effect upon the normal posture of the limb could be detected on removing its sympathetic supply. We have, however, since had one animal in which Royle's effect was continually observed. With the animal on its back, and without any anaesthesia, three limbs would frequently be held in rigid extension, the operated limb remaining flaccid. When this leg was lifted into the extended position it would again fall away into flexion. The effect, which we observed in only one of the ten goats operated upon, was almost certainly due to trauma involved in removal of the sympathetic trunk. During the operation severe haemorrhage accidentally occurred, to stop which a pack had to be left in the cavity. When this was removed two days later inflammation had already occurred, and the goat died a fortnight later. This was the only animal in which anything approaching the results described by Royle were obtained, and there is scarcely any doubt that the effect was due to secondary injury of spinal nerves and surrounding tissues. But even in this animal the mechanism subserving tonus was intact; for with the animal quietly on its back, and with all four limbs in full flexion, we occasionally noticed in the operated limb (as may at times happen also in the normal limb), that the contraction of the tendon jerk would be maintained, so that with a succession of taps upon the patellar tendon the limb could be made to pass from full flexion to rigid extension. This only occasionally happens. Nevertheless it

shows that despite removal of all sympathetic nerves the tonic mechanism is intact, even though the limb, when passively extended, falls away from this position. As Sherrington has said, the shortening reaction "seems to graft itself upon a reflex contraction more perfectly than upon a shortening produced passively, and to be itself more perfect when elicited by active contraction than by mere passive extension of the knee under external mechanical manipulation." Decerebration experiments we have not performed on the goat, but such tests have recently been reported by Mortensen, Friedbacher and Quade (4), who could find no effects of removal of sympathetic nerves upon such rigidity, so that the goat does not seem to differ in this respect from the dog or cat. It seems to us, therefore, that what effects have been obtained in the goat are due to trauma accompanying the removing of the sympathetic trunk, and not to the absence of the sympathetic nerves themselves.

SUMMARY.

1. In the brachial plexus of the pigeon the sympathetic ganglia are so intimately associated with the spinal nerves that postural effects following upon removal of these ganglia may be ascribed to unavoidable injury of spinal nerves, and not to removal of the ganglia alone.

2. The histological structure of the interosseus muscles of the dog is not visibly affected by deprival for a period of a year of the sympathetic supply, nor is there a change in thickness of the fibres.

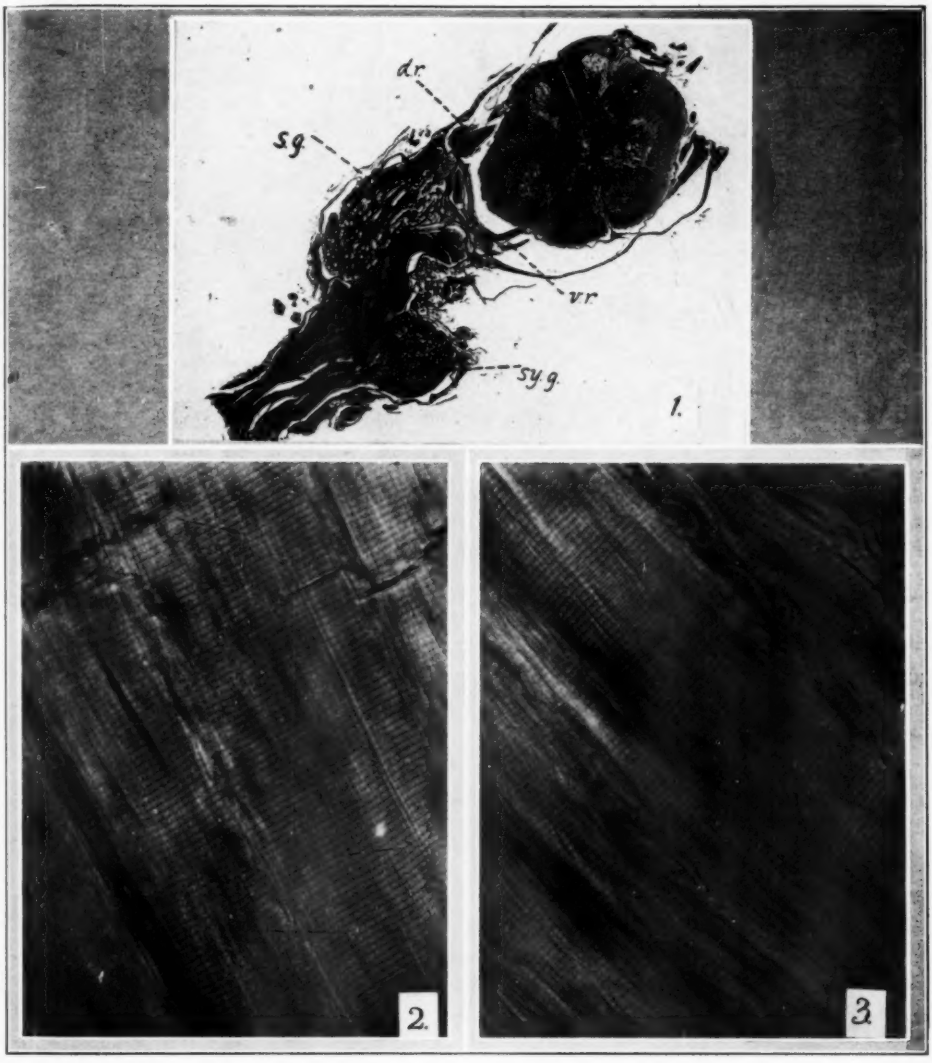
3. Contrary to Royle, we find that the sympathectomized limb of the goat does not behave differently from the normal limb to injections of ephedrine into the jugular vein.

4. In the goat apparent lack of tone in the sympathectomized limb (when such occurs) seems to be due to trauma, and not to removal of sympathetic nerves.

In conclusion, we wish to express our thanks to Dr. E. S. J. King for preparing the microphotographs that illustrate this paper, and to Dr. H. E. Albiston for the accommodation of our animals in the Veterinary Institute.

REFERENCES.

1. Coates, A. E. and Tiegs, O. W.: Aust. Journ. Exper. Biol. and Med. Sci., 5 (1928), p. 9.
2. Gaissinsky, B. E. and Lewantowsky, M. I.: Journ. Physiol. (Proc. Physiol. Soc.), 67 (1929), p. v.
3. Hunter, J. I.: Brit. Med. Journ., 1 (1925), pp. 179, 251, 298, 350, 398.
4. Mortensen, O. A., Friedbacher, K. F. and Quade, R. H.: Proc. Soc. Exper. Biol. and Med., 25 (1928), p. 757.
5. Popa, F. and Popa, G.: Journ. Physiol. (Proc. Physiol. Soc.), 67 (1928), p. 1.
6. Royle, N. D.: Med. Journ. of Aust., 1 (1929), p. 740.



EXPLANATION OF PLATE.

Figure 1. Transverse section of spinal cord of pigeon at level of third brachial nerve (C.13), to show association between sympathetic ganglion and somatic nerve. The bony vertebra has been broken away. d.r. dorsal root; v.r. ventral root; s.g. sensory ganglion; sy.g. sympathetic ganglion.

Figure 2. Muscle fibres from interosseus muscle of dog, deprived for one year of its sympathetic connections. Formalin-glycerine, unstained.

Figure 3. Similarly prepared muscle tissue from the opposite (unoperated) foot.

ON A SIMPLIFIED METHOD FOR THE DETERMINATION OF BLOOD CHOLESTEROL

by

EMILY M. DAY AND ADOLPH BOLLIGER

(From the Department of Biochemistry, Royal Prince Alfred Hospital, Sydney, and the Research Laboratory, Department of Urology, University of Sydney).

(Submitted for publication 11th April, 1930.)

The determination of the blood cholesterol in clinical investigations is of growing importance. Its great significance in physiology and pathology has been very recently summarized by Chamberlain. The gravimetric determination of cholesterol as cholesterol digitonate (Gardner) is too cumbersome for routine clinical use. The usual well-known colorimetric methods of Bloor, Myers and Wardell, Leiboff and others apply the Burchardt-Liebermann reaction, i.e., addition of acetic anhydride and sulphuric acid to a chloroform extract of the blood. Myers and Wardell and Leiboff extract cholesterol from blood which has been dried with plaster of Paris or on filter paper by means of extraction with chloroform under a reflux condenser; Bloor, on the other hand, makes a preliminary ether-alcohol filtrate, and extracts the residue from the evaporated filtrate by adding successive small portions of warm chloroform.

From the point of view of the rapid determination of a series of blood cholesterols the following objections may be raised against the methods mentioned above:

All these methods are more or less complicated and time-consuming.

Several of them require special apparatus, specially prepared chemicals, and special care, and in the case of direct chloroform extraction with ordinary laboratory equipment one or at most two determinations can be made at one time.

Bloor's method requires large amounts of redistilled alcohol and ether.

Also unless special care is taken with cleaning and drying the glassware the colours of the unknown and standard frequently do not match, giving results which are more than questionable.

For some time we have been using a modified and simplified method of extraction which eliminates these difficulties to a large extent.

One cc. of blood or plasma is spread on two filter papers, No. 40 Whatman, 7 cm. diameter, and is dried in air, incubator, or paraffin stove; drying at room temperature is sufficiently complete in about two hours. The filter paper is then folded and put in a dry, clean test tube 6 in. x $\frac{3}{4}$ in. Chloroform is added, sufficient to keep the filter paper well covered. Chloroform B.P. is sufficiently pure. Then the test tube, or tubes if several determinations are being done, are put in a beaker or tin, containing carbon tetrachloride up to about the same level as chloroform in the test tubes. This carbon tetrachloride bath is then put into a water bath and heated till the chloroform boils gently. Should the carbon tetrachloride start to boil, then reduce heating. The boiling of the chloroform is perfectly controlled by this means. Boil for about 15 minutes, and should the chloroform evaporate down to the filter paper add more chloroform. This chloroform extract, which is always colourless, is poured into a graduated or volumetric flask and made up to known volume, most conveniently 15 cc., with several chloroform washings of the filter paper in the test tube. Somewhat more complete extraction is obtained by boiling the first washing for a few minutes. Then proceed to estimate cholesterol in an aliquot of the extract, preferably 5 cc., by adding 2 cc. acetic anhydride and 0·1 cc. concentrated sulphuric acid as described by Myers. The standard solution is similarly prepared, e.g., 1 cc. of a stock solution containing 0·1% cholesterol is made up to a volume of 15 cc. with chloroform. Allow the mixture to stand for 10 minutes in the dark, and compare in the colorimeter.

Calculation: $X = \frac{\text{Standard}}{\text{Reading}} \times 100$ mgrs. % cholesterol. Where not much blood is available, 0·2 cc. of blood is sufficient to give satisfactory colouration for the determination. In such cases one only uses half of one of the filter papers, and the extraction is done conveniently in a narrower test tube and with about 2 cc. of chloroform. The final volume, including washing, is made up to 5 cc., and acetic anhydride and sulphuric acid are added to this, and a standard prepared, as in the procedure described above.

The results as shown in Table 1, obtained with 0·2 cc. of blood, agree closely with those obtained with 1 cc. of blood or plasma. This method was found suitable for use with finger-tip blood.

$$\text{Calculation}: X = \frac{\text{Standard} \times 5 \times 100}{\text{Reading} \times 3} \text{ mgs. \% cholesterol.}$$

Using 0·2 cc. of blood, drying of the filter paper may be omitted in cases where an immediate determination is required. In the case of the determination with 1 cc. of blood or plasma we preferred to use drying, which is easily accomplished by exposing the filter paper, clamped in an artery forceps, at room temperature.

TABLE 1.

Cholesterol found by the Method of Myers and Wardell. Mgrs. %	Cholesterol found by the Author's Method. Using ·2 cc. blood. Mgrs. %	Using 1·0 cc. blood. Mgrs. %
168	166	166
161	158	161
164	166	156
212	210	201
404	388	382
347	341	341
75	73	75

DISCUSSION.

Several methods for the extraction of cholesterol from blood have been described in the literature; we consider ours to be the simplest and most easily adapted to clinical laboratories. In regard to equipment nothing but an ordinary test tube is required, which obviates the discolouration brought in by a more complicated apparatus, which is difficult to clean and dry, and also the use of cork and rubber connections. This is probably the reason that in several hundred extractions of plasma, as well as of whole blood, we have never encountered a discolouration. We have found it unnecessary to use specially purified chloroform.

Table 1, showing a comparison of results obtained by the percolation method of Myers and Wardell, and by our extraction method, shows a close agreement between the two methods. In both cases the extraction is not mathematically complete, and our results have a tendency to be slightly lower than those obtained by percolating for half an hour. From the point of view of clinical cholesterol determination this objection is immaterial. Pathological changes are of great magnitude, and errors within the limits of $\pm 5\%$ are of no consequence. As seen in Table 1, the difference between the percolation and extraction methods are less than $\pm 5\%$. We have had no opportunity to check the method against the digitonin method.

The use of two thin filter papers unquestionably facilitates a speedy and practically complete extraction.

The extraction in the test tube could be done directly in a water bath, but to avoid the danger of bumping of the chloroform we place the test tubes in a vessel containing carbon tetrachloride, which has a boiling point of 78°C., sufficiently close to the boiling point of chloroform (61°C.) to prevent bumping of the latter, which can be kept boiling quietly without too much evaporation.

There is no limit to the number of extractions that can be carried out at one time. But in the final comparison with the standard no more than about eight specimens should be used at one time, on account of the rapidity with which the colours fade.

CONCLUSIONS.

1. A simplified method of extraction of cholesterol from blood and plasma dried on filter paper has been described.

2. Results with the new method compare favourably with those of standard clinical methods now in use.

REFERENCES.

1. Bloor, W. R.: Journ. Biol. Chem., 24 (1916), p. 227.
2. Chamberlain, E. N.: Brit. Med. Journ., (1929), p. 885.
3. Gardner, J. A. and Fox, F. W.: Biochem. Journ., 18 (1924), p. 1058.
4. Leiboff, S. L.: Journ. Biol. Chem., 61 (1924), p. 177.
5. Myers, V. C. and Wardell, E. L.: Journ. Biol. Chem., 36 (1918), p. 147.

FACTORS AFFECTING THE DEPOSITION OF DENTAL CALCULUS[1]

by

GEOFFREY H. SMITH

(From the Biochemical and Dental Research Laboratories of the University of Melbourne).

(Submitted for publication 16th May, 1930.)

INTRODUCTION.

The problem confronting investigators into the subject of dental calculus is to explain the cause and nature of the deposition and to elucidate the factors which render some individuals susceptible to very rapid calculus formation, whilst others, living under approximately the same conditions, are entirely free from this condition. Clinically two types of dental calculi are recognized.

1. *Salivary Calculus.* This form is light in colour, is deposited quickly, and is easily removed. The commonest situations are at the lingual side of lower incisors, and the buccal side of upper molars, near the orifices of Wharton's and Stenson's ducts respectively. Though these sites are the most common, it may be deposited in any situation, its deposition being favoured by lack of cleanliness, irregularities of the teeth, lack of mastication due to sensitive or unopposed teeth, or by any condition that causes saliva and food debris to collect and remain undisturbed.

This form of calculus is also commonly deposited on artificial dentures, and most commonly again near the orifices of the salivary ducts.

2. *Subgingival Calculus* (also known as serumal or haematogenic calculus on account of its supposed source of origin). This form of calculus is found beneath the free margin of the gum; it is dark in colour, is formed slowly, and is firmly adherent to the teeth. Sometimes it is found in thin flakes well down the roots of the teeth, in places where there is apparently no access to the saliva.

Dental calculus is extremely variable in composition, no two analyses being the same. Prinz (30) gives as an average of five analyses the following percentage composition:

---

[1] This work was done during the tenure of a Fred Knight Research Scholarship of the University of Melbourne, and formed a thesis for the Degree of Doctor of Dental Science.

Water	5·68
Water-soluble substances	5·24
Organic constituents (mucin and food debris)	15·16
Magnesium phosphate	1·31
Calcium carbonate	9·14
Calcium phosphate	63·42

Stevenson and Schehevetskey found a greater amount of inorganic material, and less organic, in the hard or subgingival variety of calculus. The results of their analyses are as follows:

	Soft Tartar.	Hard Tartar.
Water and organic matter	21·48	17·51
Magnesium phosphate	1·31	1·31
Calcium phosphate with a little carbonate and a trace of fluoride	77·21	81·18

The variability in the amounts of the respective constituents is due partly to the variability in the composition of saliva, and also to the different amounts of food debris and other foreign materials incorporated in the calculus. Badanes (3) has recently shown calcium oxalate to be present in addition to the above constituents, especially in subjects whose diet contains articles such as rhubarb, spinach, asparagus, etc., which are rich in oxalates. Adamson (1) demonstrated the presence of cholesterol in a mixed sample of tartar, and is of the opinion that it occurs only in serumal calculus. The present writer, however, has found cholesterol in a sample of salivary calculus, and also in saliva itself. In the saliva, the cholesterol is contained wholly in desquamated cells, which are always present in varying amounts, and which become incorporated in the salivary calculus along with mucin and other debris.

Dental calculi consist of calcium salts, food debris, epithelial cells, and organisms held together in a matrix of mucin. Badanes (3) is of the opinion that globulin also plays an important part as an initial matrix.

Opinion is rather divided as to whether lamination occurs in dental calculi. Burchard (9) was of the opinion that lamination occurs, and Prinz (30) states that, if the deposition is not interfered with, concentric lamellae are formed, but in most cases the precipitation occurs in more or less irregular masses. Mechanical causes, such as chewing, movements of the tongue and cheeks, the use of the toothbrush, etc., tend to disturb the deposition, and as it takes years for a large specimen to form, the "onion layer" deposits are rarely sufficiently defined to be observed with the naked eye. However, with a magnifying glass the layers, irregular as they may be, can usually be made out. Prinz also quotes Wedl, who found irregular wavy strata on the polished surfaces of dental calculi. Hulin (18) also found that after decalcification of dental tartar stratified zones were visible.

On the other hand, Adamson (2) is of the opinion that dental calculi are practically homogenous, and do not show a laminated structure. The present writer had the opportunity of examining the same sections upon which Adamson based his opinion, and agrees with him regarding them. It is probable,

however, that large specimens, which have taken years to form, would, when carefully ground and polished, always show lamination to some extent, but never in the well-defined manner which is seen in some gall stones, for example, owing to the very variable conditions occurring in the mouth.

GENERAL CONSIDERATIONS.

In discussing the possible factors influencing the formation of dental calculi, it must be borne in mind that dental calculi are not essentially different in their mode of formation from calculi in other parts of the body. It is generally recognized that two conditions are necessary for the formation of any calculus. Firstly, there must be a nucleus, which may be composed of desquamated cells, bacteria, precipitated proteins, or a foreign body. Secondly, the fluid or biological medium, from which the calculus is formed, must be supersaturated in respect to the substance which is going to constitute the calculus.

The rôle played by the nucleus may be primary or secondary, i.e., its presence may be the cause of the deposit, or the salts forming the deposit may be thrown down independently, and then, finding a suitable nidus in one or more of the above-mentioned forms, adhere to such, and form the beginning of a calculus. The fundamental factor in dental calculus formation is the precipitation of calcium phosphate from the saliva, and in the case of serumal calculus, the serum also is concerned.

It is therefore necessary to consider the saliva with special reference to its calcium phosphate content, and the possible factors tending to cause its precipitation. Calcium phosphate is present in saliva in amounts which normally would produce a supersaturated solution in water. Similar conditions exist in blood serum and urine.

Urine does not accurately follow the laws of supersaturation in aqueous solution, since a clear specimen of urine may contain some constituent in amounts far in excess of the quantity soluble in water, yet no crystallization will follow the suspension in the urine of a crystal of the substance. Saliva probably behaves in a similar way. This phenomenon may be explained by the fact that a crystalloid may, under certain conditions, exist in a form of suspension, the particles being exceedingly small, but always larger than a molecule, i.e., in a colloidal solution. This property is not inherent in the substance itself, but is due to outside influences. Calcium phosphate is present in this condition in saliva, and the maintenance of this state of colloidal suspension is due to the protective action of the colloids of the saliva, i.e., mucin and globulin.

Another factor which is undoubtedly important in holding at least some of the calcium phosphate in solution is the carbon dioxide present in the saliva.

Saliva contains from 40–50 volumes per cent. of carbon dioxide, and the amount of calcium phosphate in solution can be markedly lowered by removing this carbon dioxide.

It is believed by Barillé, and also by Gassman and other investigators, that carbon dioxide and calcium phosphate form a definite compound which they term a carbon-phosphate of calcium. This compound is very unstable in solution, a reduction of pressure resulting in the splitting up of the compound, with precipitation of calcium phosphate and evolution of carbon dioxide.

THEORIES OF FORMATION OF DENTAL CALCULUS.

1. *Loss of Carbon Dioxide.*

Kirk (21) was the first to offer the hypothesis that the escape of carbon dioxide from the saliva resulted in a precipitation of calcium salts. He states that "as the saliva contains CO_2 in solution, it has been assumed, with some justification, that the escape of CO_2, which is the solvent of the calcium carbonate and tricalcic phosphate, causes a precipitation of these salts in the presence of the colloid mucin."

Kelsey (20) also states: "Entering the mouth the saliva meets the acids and ferments derived from the decomposing food debris, and with the atmospheric oxygen. This causes CO_2, which is in very unstable solution, to be liberated. The saliva then loses its power of holding the salts in solution, and they are precipitated."

Naeslund (26) advances some conclusive arguments against the loss of CO_2 as a likely cause of calculus formation. He states: "Experimental evidence has shown that, *in vitro*, no perceptible precipitation takes place until sufficient CO_2 has escaped to bring the pH to over 7·4. . . . . Investigations on the pH of saliva of people susceptible to tartar have shown that the escape of CO_2 from the oral cavity is not sufficiently great to result in an appreciable precipitation of calcium." He further adds that the loss of CO_2 occurs in all persons, and must be quite a normal process, while tartar occurs only in some.

The present writer has also performed experimental work which shows that as a factor in calculus formation the loss of CO_2 is of very slight importance.

First of all it was thought advisable to see whether calcium phosphate could be precipitated from saliva by exaggerating the conditions met with in the mouth. A 20 cc. sample of saliva was saturated with CO_2 by bubbling the gas through the saliva for about 10 minutes. This was done to ensure the solution of any calcium salts that might have been precipitated through loss of CO_2 when collecting the saliva. 10 cc. were then filtered and total phosphate estimated in 2 cc. of the filtrate by the method described later. The other 10 cc. were pipetted into a wide test tube, which was then placed in a vacuum flask connected with an exhausting pump. The pressure was reduced till no

more CO_2 was evolved. After standing for 45 minutes the saliva was filtered and total phosphate estimated in the filtrate as before.

Results (expressed in mgs. phosphorus in 100 cc. saliva):

Before removal of CO_2	20·30 mgs.
After „ „ „	18·10 mgs.

The results indicate that there was less phosphate in the filtrate of the saliva from which the CO_2 had been removed. This must be due to a precipitation of calcium phosphate from solution, as it is the only phosphate that could be rendered insoluble by the treatment received.

The above experiment was then repeated, and in addition calcium was estimated in the filtrate by the method of Kramer and Tisdall.

Results (in mgs. per 100 cc.):

	P.	Ca.
Before removal of CO_2	17·38	7·54 mgs.
After „ „ „	15·62	2·8 mgs.

This experiment shows that calcium phosphate is precipitated in one or other of its forms. The conditions of this experiment, however, were certainly not similar to those occurring in the mouth.

The following experiments were performed in an attempt to see whether a similar precipitation could be brought about under conditions more closely approximating those in the mouth, and instead of completely exhausting the CO_2 a mixture of CO_2 and air, containing 4% CO_2, about that of expired air, was bubbled through the saliva for 20 minutes. Calcium and phosphate were estimated in the filtrates as before.

The following table shows the results of three such experiments, the figures representing mgs. per 100 Ca saliva. In experiments 2 and 3 the precipitation caused by bubbling through air freed from CO_2 was also determined.

	P.	Ca.
1. Original	18·78	7·35
After 4% CO_2	17·62	6·31
2. Original	20·40	7·64
After 4% CO_2	20·20	7·16
After CO_2—free air ..	17·94	4·62
3. Original	17·24	7·79
After 4% CO_2	17·10	7·24
After CO_2—free air ..	15·74	3·26

The results of this experiment show that a large amount of calcium phosphate can be precipitated from saliva by complete removal of CO_2, but that only minute amounts are precipitated when the saliva is exposed for some time to air containing 4% CO_2. The fact that only traces of calcium phosphate are precipitated from saliva, under conditions much more favourable for precipitation than ever occur in the mouth, seems to show that loss of CO_2 from saliva is of little importance as a cause of dental calculus deposit.

2. *Physico-Chemical Theory.*

A theory which has received much support recently is concerned with a physico-chemical phenomenon.

Prinz (30) is responsible for this view as applied to saliva. Quoting *verbatim:* "The process occurs in conformity with the laws governing the precipitation of colloidal solutions as previously discussed, and essentially depends upon the harmonious co-ordination of three basic factors:

1. The presence of a supersaturated solution of calcium salts in a colloidal medium, i.e., saliva.
2. Its stagnation in a sheltered place.
3. A parenteral or foreign inanimate substance having a hard rough surface, which serves as a nucleus for its attachment.

. . . . The stagnated saliva in conformity with Gibbs' law of surface tension concentrates its colloids upon its own surface. At first a faint nebula appears which by its own concentration slowly forms a dull lustrous pellicle. Simultaneously, the solvent power of the saliva is thereby physically diminished, thus liberating the crystalline calcium salts which are at once caught by the fully formed colloidal stroma. The pellicle becomes gradually specifically heavier than the surrounding fluid, and slowly sinks or is forced along the line of least resistance by the movements of the cheeks, lips, and tongue, till it meets with some obstruction. Any sheltered surface which offers a suitable rough focus for its attachment, where the film can remain undisturbed, may serve as a nucleus for its permanent retention."

A somewhat similar process is generally supposed to occur in the urine, which is also a colloidal suspension. In any such colloidal suspension the suspended particles tend to accumulate at any point where the surface tension is increased. Normally the surface tension between urine and normal mucosa is practically nil. In contact with air, glass, foreign bodies, etc., the surface tension is raised, and an accumulation takes place illustrated by the pellicle which often forms when urine stands in contact with the air. When the character of the foreign body is such that little or no increase of surface tension is caused, e.g., a clean piece of pure paraffin, no urinary stone formation occurs.

It, indeed, seems feasible that similar principles should also apply to the formation of dental calculi, but the amount of experimental evidence is not conclusive. The experimental work which Prinz carried out to demonstrate these principles was as follows: "A small test tube was completely filled with fresh saliva. Within about an hour the formation of the pellicle could be observed at its surface, which finally would sink slowly to the bottom of the tube, and which was followed by additional pellicles." These pellicles as they

form are supposed to entangle the calcium phosphate which is thrown out of solution, and carry it to the bottom of the tube as they sink.

If such a process occurs, one would expect a decrease in the calcium phosphate in the upper layers and an increase in the lower layers. The author therefore repeated the experiment of Prinz several times, allowing the saliva to stand undisturbed for 20 hours. On observation a slight scum was noticeable on the surface, showing diffraction colours, but the tendency to sink to the bottom of the tube was not apparent. The free phosphate was then carefully estimated in the uppermost and lowermost portions of the tube, care being taken not to disturb the saliva when using the pipette. In all cases the figures obtained were, within the limits of experimental error, exactly the same for both upper and lower portions. The author does not wish to put too much stress on the result of this experiment, but would point out that the theory of concentration of colloids as applied to the formation of dental calculi is, after all, only a theory, and, plausible as it may be, it is extremely difficult to prove or disprove experimentally.

Badanes (3) suggests, as a slight modification of the above theory, that the deposition is more rapid and due to the precipitation of the globulin of saliva by water or by salty foods. The precipitation of the colloid, together with the calcium phosphate, takes place instantaneously, and even in places where the saliva is not stagnated. The film of liberated globulin, together with mucin and earthy phosphates, may act in sheltered places about the teeth as an initial matrix for succeeding layers of calcareous deposits. This view again is purely theoretical, and has no experimental evidence to support it.

3. *The Relation of Diet to Dental Calculus Formation.*

It has been recognized for a considerable time that diet has a marked influence on the formation of dental calculus, but just how diet produces the effect has never been satisfactorily determined. Black (5) during his extensive researches on salivary calculus was struck by the fact that the deposit of calculus was of a paroxysmal nature. These periods, during which a marked deposit occurred, usually followed a large meal, and he considered the kind of food was not the important factor, but that almost any good nutritious food, if taken in excessive amount, would cause a paroxysm of deposit.

It is, indeed, a common observation that people who are excessively fat, take no exercise, and are gross eaters, are often very prone to dental calculus. The problem is, therefore, to find out in what way diet modifies the salivary secretion so that it more readily precipitates calcium phosphate.

(*a*) *The Influence of Diet on the pH of Saliva.*

It appeared to the writer that the most likely change favouring a deposit of calculus would be a rise in the alkalinity or pH of the saliva so that calcium phosphate would more readily precipitate.

It has been suggested by Badanes that conditions which lead to a precipitation of calcium phosphate in the urine also have a similar influence on the saliva. This precipitation of phosphates in urine can occur as a purely physiological reaction, and is in these cases caused in two ways:

(1) A diet rich in alkali carbonates, or alkaline salts of vegetable acids, and alkaline albuminates, both of which yield alkaline carbonates on combustion in the body, leads to diminution of the acidity of the urine. One result is a transformation of some of the soluble acid phosphates, present in solution in the urine, into insoluble phosphates which are precipitated. There is actually no increase in the amount of calcium phosphate excreted, and, in fact, an experimentally increased supply of phosphate of calcium does not produce the condition.

(2) An increased alkalinity of the urine may also be due to a decreased excretion of acids. This occurs normally after the secretion of hydrochloric acid in the stomach after a meal. Baehr (4) states that: "During the period of digestion there is a temporary loss of acid into the stomach, and in order to maintain its approximate neutrality the body must therefore compensate by excreting less acid in the urine."

Diet has a marked influence on this normal urinary alkaline tide. Kaye (19) has shown that protein excites a marked alkaline tide, whilst carbohydrate is associated with an acid urine, corresponding with a large flow of HCl into the stomach in the former case, and a small flow in the latter.

Thus, it is seen that an increased alkaline tide in the urine may be brought about normally in two ways: by ingestion of foods rich in alkalies, and by ingestion of foods which are strong gastric stimulants.

The statement of Black that an increased deposit of salivary calculus follows after a rich, heavy meal, suggests the possibility of an alkaline tide in saliva, corresponding with the urinary alkaline tide. With the object of determining whether this occurs, experiments were performed by the writer, in which the subject was given meals consisting of:

(i) Strong gastric stimulants, e.g., meat, meat extract.

(ii) Meals to which were added sodium bicarbonate and sodium citrate.

(iii) Weak gastric stimulants, e.g., carbohydrates.

The pH of the saliva and urine was determined at intervals.

Determinations of hydrogen ion concentration were made, using the double quinhydrone electrode method.

The saliva was collected by expectorating into a thistle funnel, which extended beneath the surface of a few cc. of paraffin oil placed in a test tube. Loss of CO_2 was thus prevented.

Care was taken in collecting the saliva to use no means of stimulation which would be likely to cause a rise in pH. The saliva was allowed to collect in the mouth without movement of tongue or cheeks, and when sufficient volume had collected it was ejected into the thistle funnel.

For convenience the saliva was diluted before making the determinations, but first of all the effect of different dilutions on the pH had to be considered. The following experiments were therefore carried out: 30 cc. of saliva were collected under oil. In a series of nine test tubes were placed quantities of freshly boiled distilled water (pH 6·2), as set forth in the following table. To each was added the quinhydrone, and the surface then covered with oil. With pipettes, varying quantities of saliva were introduced beneath the surface of the oil to give the required dilutions. The pH determinations were then made, using the bright platinum electrode, which was immersed beneath the oil, to mix thoroughly the saliva with the quinhydrone. The table shows the results of two such experiments.

Saliva.	Water.	A. p.H.	B. p.H.
10 cc.	0 cc.	6·83	6·94
5	5	6·83	6·90
3	9	6·83	6·95
2	10	6·87	6·95
2	14	6·85	6·95
1	9	6·85	6·99
1	12	6·85	6·99
1	15	6·85	6·99
1	20	6·85	6·99

These results show that the pH of saliva is altered only very slightly by diluting up to 1 in 20, showing that saliva is well buffered. For convenience a dilution of 1 in 10 was used in all determinations.

Diet Experiments. Specimens of saliva were collected, commencing at 7.30 a.m., before rising from bed, the next sample at 8 a.m., just before breakfast, and again immediately after breakfast. From then on samples were obtained every half-hour. Urinary samples were obtained every hour, commencing at 8 a.m.

(1) A breakfast, consisting wholly of cooked meat, with a small piece of dry toast, was taken. The pH curves of urine and saliva during the morning are represented in figure 1, continuous lines.

It is seen that a marked urinary alkaline tide was produced. The saliva showed a steady but slight rise in pH from 10 a.m. onwards, but it did not correspond with the rise of alkalinity of the urine.

(2) As a modification of the foregoing experiment, a large amount of meat extract was added to the morning meal. A still greater rise in urinary pH occurred, but the curve for the pH of saliva showed no corresponding rise. (See figure 1, broken lines.)

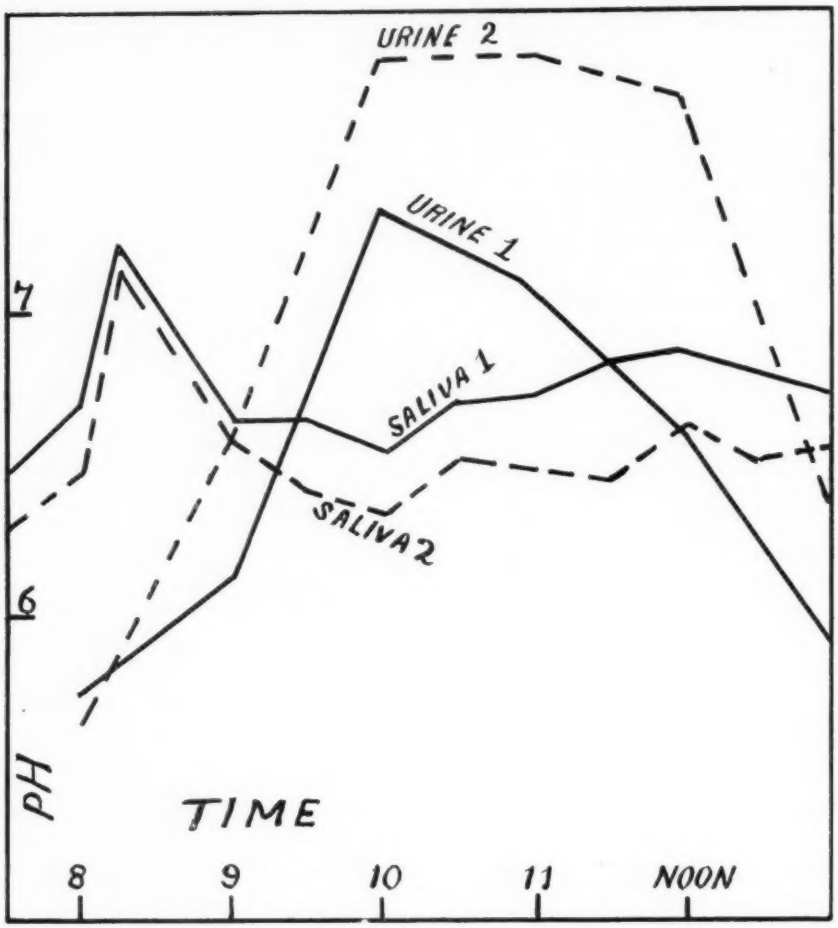


Fig. 1.

(3) In this experiment the alkaline tide in the urine was produced in the other way, i.e., by ingestion of alkalies, 5 gms. of $NaHCO_3$ being taken in addition to a protein breakfast. An enormous urinary alkaline tide was thus produced, rising to above pH 8, when a slight turbidity of the urine, due to precipitated phosphates, was observed. The salivary pH, however, was not influenced, unless, perhaps, in the direction of greater acidity. (Figure 2, continuous lines.)

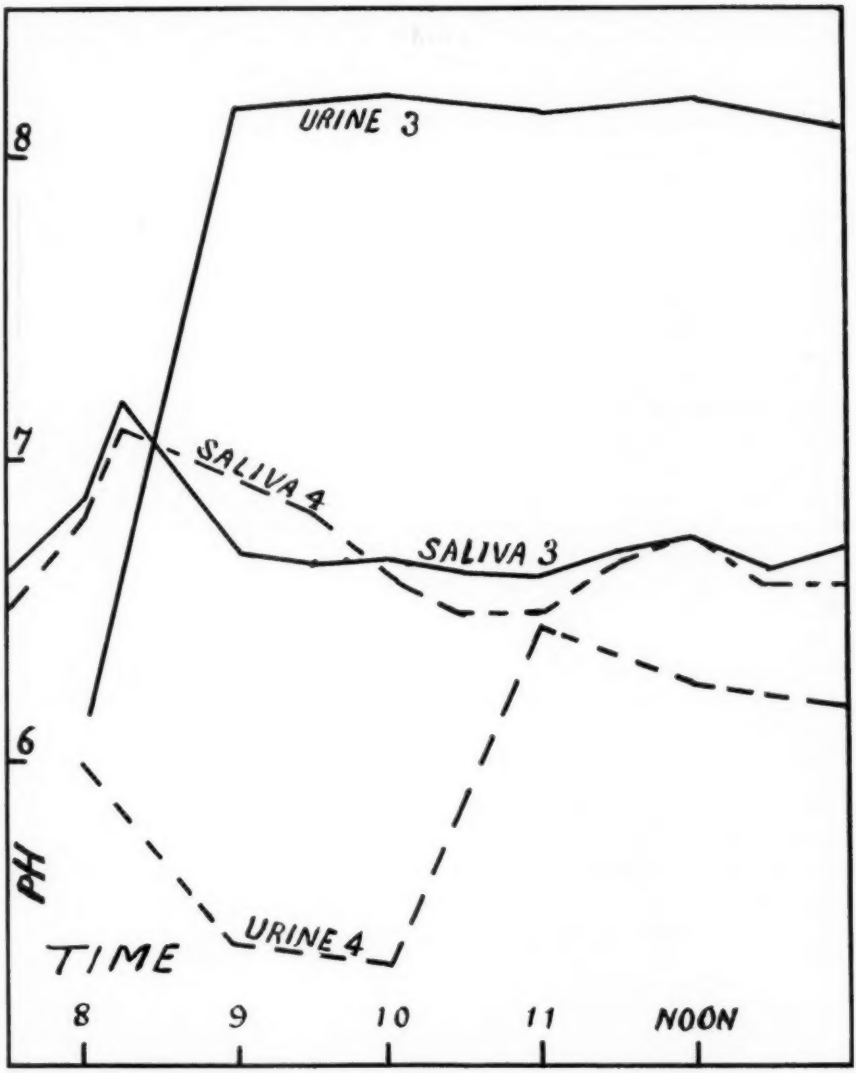


Fig. 2.

A similar result was obtained by Starr (32), who states: "The pH of saliva varies inversely with the pH of the urine after ingestion of large quantities of $NaHCO_3$." Bloomfield and Huck (6) also found that the internal administra-

tion of alkali did not influence the pH of saliva in any definite manner. Curves similar to those obtained in the last experiment were produced by ingestion of sodium citrate, which yields sodium carbonate on combustion in the body. This experiment was performed twice, and in each case the urinary pH rose to about 7·5, while that of the saliva did not seem to be influenced in any way. The curves obtained are not shown.

(4) By means of a breakfast consisting wholly of carbohydrate, calculated to produce little secretion of HCl in the stomach, an acid urine was produced, but here again the saliva was not influenced. The results are shown in figure 2 (broken lines).

The curve for saliva was very similar to those obtained in the previous experiments, while the urinary pH remained well on the acid side. The experiment was repeated with the same result.

The results obtained in this series of experiments show that the pH of saliva is not influenced by the ingestion of different types of diet; also that the pH of saliva does not correspond with changes of urinary pH, which can be readily produced by such alteration of the diet.

It was in addition interesting to note that in every experiment the pH curve for saliva took approximately the same course whether the urine was alkaline or acid. On awakening it was low, it rose slightly on arising, and reached a peak immediately after breakfast. Thence there was a sharp fall, followed by a more gradual one, till a minimum was reached in from $1\frac{1}{4}$ to $2\frac{1}{2}$ hours after the meal. From this lowest point there occurred a slight but definite rise to a maximum about 4 hours after breakfast, after which a fairly constant value was observed. After luncheon a similar sharp rise occurred, followed by a fall to a minimum, and then slowly up again to a fairly constant value. In all nine experiments were carried out, and a similar result was obtained in every case.

A very similar curve for the pH of saliva was found by Henderson and Millet (16), who also found that the alkalinity immediately after meals was not due to any particular kind of food, but that the masticatory movements themselves produced the rise in pH, even with an empty mouth. The same rise can be produced by chewing paraffin wax.

(b) The Influence of other Stimuli on the pH of Saliva.

Numerous attempts have been made to show a relation between the pH of saliva and the type of stimulus introduced into the mouth. If any one type of substance introduced into the mouth caused an alkaline saliva to be secreted, the persistent use of the same might render the deposition of calcium phosphate more likely. Numerous investigators have tried to show some such relationship. Thus acid and alkaline mouth washes and dentifrices, different types of

foodstuffs, etc., have all been tried, but no general conclusion has been reached as to the changes induced by any particular stimulus.

Pickerill (29) was of the opinion that acid substances introduced into the mouth resulted in a flow of alkaline saliva. His results, however, have not been confirmed.

McClelland (23) has shown that mastication increases pH regardless of the flavour of the material chewed, and that substances requiring vigorous chewing had more influence than soft materials. The same author (24) also showed that dentifrices, either acid or alkaline, do not change the reaction other than very slightly, and then only when some of the dentifrice is actually present in the mouth. Bunzell (8) and Carlson and McKinstry (11) have obtained similar results.

The case of extreme tartar deposition in betal nut chewers, as cited by Kirk (22), is an example of the alkalinity of the saliva being kept abnormally high by the frequent introduction of lime into the mouth. These cases, however, cannot be compared with those of people who are susceptible to dental calculus, living under the same conditions and eating the same food as other people who are completely immune from calculus.

From the evidence it is therefore probable that the pH of saliva does not show large enough variations from the normal to exert any marked influence on calculus formation. It has been shown by Naeslund (26) that *in vitro* a pH of over 7·4 is necessary to bring about a precipitation of calcium phosphate, and investigations on the salivary pH of people susceptible to tartar have shown that the pH does not become high enough to result in an appreciable precipitation of calcium from this cause alone.

Starr (32) found that the average value for the salivary pH in 610 cases was 6·6. Bunzell (8) found an average of 6·4 in 324 cases.

It must be remembered, however, that immediately after meals the pH of the saliva rises well above 7·0, and although this degree of alkalinity is not maintained for long, it may be during this period that the conditions for precipitation of calcium salts are most favourable.

(*c*) *The Calcium Content of Saliva as Related to Diet.*

It is necessary to consider any other means by which the saliva could be modified by diet so as to render it more liable to deposit calcium phosphate. It is natural to suppose that saliva of high calcium content would more readily deposit calcium phosphate than saliva containing only a small amount.

Numerous dietetic experiments have been performed in attempts to modify the amount of calcium in saliva. Clark (12) carried out elaborate experiments, in which the ingestion, excretion, and retention of calcium, magnesium, sodium, and phosphorus were definitely known over a period of 28 weeks. He con-

cluded that there was no relation between the amount of a given element ingested and the amount appearing in the resting saliva.

It must be remembered, when carrying out experiments of this nature, that it is extremely difficult to prove whether the ingestion of substances actually causes any alteration in the composition of the saliva because of the wide range of variations that occur naturally. This is probably due to the fact that the secretory functions are dominated by a very complex nervous mechanism, and the secretion and composition may therefore be altered by a large variety of uncontrollable stimuli.

Thus Clark, and also Payne (28), found that the calcium content of resting saliva was subject to large variations, not only in different subjects, but also in weekly values for the same individual. In addition, Clark found that the values for calcium vary even more during fasting periods.

All investigators are of the opinion that the calcium content of saliva cannot be increased by increasing the calcium content of the food over a short period. Thus Clark increased the Ca content of the food by 28·53% for seven weeks with no effect on the salivary content. Similarly the daily ingestion of 3 gms. of calcium lactate for two weeks had no effect.

Of particular interest in this connection is the work of Pattison (27), who was able to bring about large increases in salivary calcium by a diet rich in fat-soluble vitamins. On the other hand, a low Ca content was produced by a diet containing much cereal (specially oatmeal) and comparatively little fat-soluble vitamins, although the amount of calcium ingested may be the same or even greater than in the first case.

These experiments were performed on tuberculous children, who were decidedly deficient in salivary calcium to begin with, and it is doubtful whether similar diets would affect the Ca content of the saliva of adults in the same way.

In the light of the evidence it cannot be said that the calcium content of the diet has any influence on the Ca content of saliva, and indeed it has not yet been proved that the saliva of people susceptible to calculus contains more calcium than the saliva of those who are immune; on the contrary, Bunting and Wixon, quoted by Clark and Levine (13), found a normal calcium content in the saliva of patients showing much tartar.

4. *The Rôle of Organisms in Dental Calculus Formation.*

From the experimental evidence that has appeared in the literature in the last few years, it is evident that certain organisms may play a very important part in the production of dental calculus. It was first suggested by Goodrich and Moseley (15) that *Leptothrix buccalis* is essential for the production and deposition of tartar. They found that coarse leptothrix threads were always present in smears taken from situations in which tartar was present, and also that leptothrix threads were present in deposits of tartar on the roots of teeth that

had apparently no lesion leading to the mouth cavity. The amount of experimental evidence was not conclusive, but ten years later Bulleid (7) performed experiments in which he showed that a deposit of calcium phosphate could be brought about by growing leptothrix in sterile broth containing calcium chloride.

The work of Naeslund (26) agrees closely with that of Bulleid. He considers further that the colonies of leptothrix, and also of actinomyces, may adhere to the teeth, and themselves serve as an excellent matrix for the deposit of calcium salts. It is probable, however, that it is only when there is some damage, mechanical or bacterial, in some situation suited for the lodgment and unhindered multiplication of organisms, that these organisms can form colonies and by their action cause a deposit of calcium in the network of their own filaments.

5. *The Influence of Enzyme Action on Dental Calculus Formation.*

Robison and his co-workers (31) have in the last few years shed entirely new light on the very controversial point of the actual nature of the processes resulting in the deposition of calcium phosphate in ossifying bones. The presence of an enzyme in bones and ossifying cartilage of young animals, which can rapidly hydrolyze hexosephosphoric and glycerophosphoric esters, was demonstrated. As a result of this hydrolysis free phosphate ions are liberated.

As a further result of his experimental work it was suggested that the enzyme or phosphatase present in bone, by its action on the phosphoric esters of the blood, brings about a local increase in phosphate ions, and so causes a deposition of solid calcium phosphate in actively ossifying centres.

Adamson (1) applied Robison's theory to the problem of dental calculus formation. It was shown that a phosphatase is present in human gum tissue, and also that the saliva contains a phosphate-containing complex, probably in the nature of a phosphoric ester, which is capable of being hydrolyzed by the gum tissue enzyme with the liberation of free phosphate.

It was concluded that the deposition of serumal calculus is almost entirely due to the interaction of the enzyme and phosphoric esters of the blood serum.

With regard to the salivary type of calculus, Adamson is of the opinion that although, under suitable conditions, the action of the gum enzyme on saliva can lead to the formation of salivary calculus, it probably plays little part in the deposition of this variety in the mouth.

The present writer has carried out further investigations into the possible part played by a phosphatase in dental calculus formation, and the methods used and the experimental results are given below.

Organic Phosphate in Saliva. It was first of all thought advisable to determine the amount of organically combined phosphate present in saliva, and to see whether the presence of a large amount could be correlated with any increased tendency to calculus formation. It was previously thought that all

the phosphate in saliva was in inorganic form (Clark, 12), but Adamson showed conclusively that some at least is combined in organic form, and is capable of being set free by the hydrolytic action of a phosphatase. No accurate quantitative estimations were, however, performed.

METHOD.

The accuracy with which the inorganic and total phosphates can be estimated is of great importance, as the organic portion can only be determined by the difference.

Inorganic phosphate estimations were performed colorimetrically, but several difficulties had to be overcome before a reliable method was obtained. Brigg's method as applied to blood was found unsatisfactory in an unmodified form, as a turbidity always occurred after the addition of the ammonium molybdate to the protein-free filtrates.

Trichloracetic acid was used to precipitate the protein, and although perfectly clear filtrates were obtained, which were negative to all protein tests, the turbidity always occurred. Various strengths of trichloracetic acid and other protein precipitants were tried, but with the same result. The nature of the precipitate could not be determined, although many attempts were made. Robison and Soames encountered a similar difficulty in estimating inorganic phosphates in rats' blood, and found it to be due to the presence of protein cleavage products. If the colours were matched with the turbidity present a large error was introduced. If filtered after development of the colour, error due to adsorption on the filter paper was introduced. If the turbidity was centrifuged down after full development of the colour the values obtained were too low, showing that some inorganic phosphate was carried down in the precipitate.

The following experiment was carried out to compare the results obtained by the method finally adopted (see later), with those obtained by the method of centrifuging down the turbidity after the colour had developed fully.

Results (in mgs. P per 100 cc. saliva):

Finally adopted method.	Centrifuge method.	Difference.
16·23	14·47	1·76
14·21	12·27	1·94
14·82	16·65	1·83

These results show that on an average 1·84 mg. P per 100 cc. saliva are brought down in the precipitate caused by the addition of the ammonium molybdate.

It was therefore decided to precipitate the inorganic phosphate as magnesium-ammonium-phosphate, to take up the precipitate with acid, and

then estimate the phosphate colorimetrically. The method finally adopted was as follows:

Ten cc. of saliva were run from a pipette into 10 cc. of 10% trichloracetic acid contained in a small Erlenmeyer flask; 10 cc. of distilled water were added, and the flask well shaken. Filtration was carried out in a Buchner funnel through two fairly fine filter papers, using slight suction. 20 cc. of the filtrate were then pipetted into a 50 cc. centrifuge tube with a spout suitable for pouring. 3 cc. magnesium citrate mixture were added, followed by 5 cc. of 30% ammonia. Precipitation was initiated by scratching the side of the tube with a glass rod, and then stirring vigorously for three minutes. The tubes were usually allowed to stand overnight, after which filtration was carried out through a 9 cm. quantitative filter paper. The filtrate was put through the filter a second time, after which the precipitate was washed through into a 100 cc. graduated flask with hot dilute sulphuric acid (2·5 cc. conc. H_2SO_4 in 500 cc. dist. water), using about 75 cc. After cooling, a similar flask, containing 1 mg. phosphorus as phosphate, was used as a standard for comparison. To each flask was added ammonium molybdate and sulphuric acid, so that equal amounts of sulphuric acid and of ammonium molybdate were present in both standard and unknown.

Two cc. of aminonaphthol sulphonic acid reagent (Fiske and Subbarow, 14) were then added, followed by distilled water up to the mark. The flasks were allowed to stand for half an hour, and the colours then compared in a Klett colorimeter with the standard set at 15 mm.

As a test for the accuracy of the method, 10 cc. of the standard phosphate solution (containing 1 mg. P) were taken, and the same procedure carried out as for saliva. In duplicate estimations the amounts recovered were 1·015 and 1·02 mg. P. This was repeated several times, and the error did not exceed $\pm 2\%$, which is not large, considering the small quantities used.

Total Phosphate. 5 cc. of the original protein-free filtrate were pipetted into a micro-Kjeldahl flask, and to it added 1 cc. of a mixture of sulphuric and nitric acids (1 part H_2SO_4 to 2 parts HNO_3). The flask was heated carefully over a very small flame until dense white fumes began to come off. The flask was then cooled, and the residue transferred into a 50 cc. volumetric flask, using about six washings of distilled water. For the standard it was found that 3 cc. of a standard phosphate solution containing 0·3 mg. phosphorus gave a suitable match.

To each was added 2 cc. of the molybdate solution, that added to the unknown containing no acid, an equivalent amount being already present in the digestion mixture. 2 cc. of the reducing agent were added, and then distilled water up to the mark. Readings were taken after half an hour, with the standard set at 20 mm.

The accuracy of this method was tested with solutions of sodium glycerophosphate. 1·0767 gms. of sodium glycerophosphate (dried) were made up to a volume of 250 cc., and total phosphate estimated in 2 cc. of this solution. Duplicates gave values of 61·75 mg. P per 100 cc., or 14·33%; theoretical = 14·35%.

Experiments with Saliva. Having therefore arrived at a method in which confidence could be placed, a series of estimations of phosphate in saliva were carried out. The saliva was collected, without resorting to any means of stimulation, by expectorating into a graduated cylinder until the required amount was obtained. 10 cc. were immediately pipetted into the trichloracetic acid and the estimation proceeded with.

Results (values expressed as mgs. phosphorus per 100 cc. saliva):

Subject.	Inorganic Phos.	Total Phos.	Organic Phos.
I	19·72	21·17	1·45
,,	17·17	19·46	2·29
,,	15·09	16·00	0·91
,,	17·85	20·35	2·50
,,	23·19	25·90	2·71
,,	25·56	27·90	2·34
II	16·06	17·42	1·36
,,	15·19	17·84	2·65
,,	20·82	23·18	2·36
,,	18·75	19·57	0·82
III	19·54	20·75	1·21
IV	19·90	21·53	1·63
,,	17·44	19·15	1·71
V	9·22	10·49	1·27
VI	17·64	18·47	0·83
VII	17·64	18·85	1·21
VIII	13·80	16·56	2·76
IX	16·78	17·64	0·86

The figures obtained showed a wide range of inorganic phosphate, viz., 9–25 mgs. P per 100 cc. of saliva. The organic phosphate was from 0·82–2·76 mgs. P per 100 cc. saliva, or 5–17% of the total phosphate. The average figures for organic phosphate were 1·71 mgs., or 11·5% of the total.

The first six values were obtained from the writer's own saliva on different days, and show that considerable variations occur in both inorganic and organic phosphate. Similar results were obtained with another subject, II.

The cases studied included some that showed extreme degrees of calculus deposition, but no significant increases in either inorganic or organic phosphate could be demonstrated.

Martland and Robison (25) found similar variations in the organic phosphate of blood serum, but could throw no light on the cause of these variations. It is probable, however, that the cause or causes, whatever they may be, exert a similar influence in producing similar variations in saliva.

The inorganic phosphate can easily be increased both in blood serum and saliva by doses of phosphates, but the organic phosphate is not influenced.

It could only be concluded, therefore, that no relation was observed between the amount of organic phosphate present in saliva and a tendency to calculus deposition.

Increase of Inorganic Phosphate when Saliva is Incubated. It was found that, when saliva is incubated at 37°C. in the presence of chloroform (to prevent bacterial action), there is an increase of free phosphate. This was thought to be due to an enzyme present in the saliva itself, acting on the organic phosphate of the saliva. This supposition later proved to be correct.

In a 20 cc. sample of saliva the free and total P was estimated at once in one-half; the other half was incubated in the presence of chloroform for 20 hours at 37°C., and the free P again determined.

Results (in mgs. P per 100 cc. saliva):

	Free P before incubation.	Free P after incubation.	Increase.	Total.
a	15·00	15·41	0·41	15·83
b	25·56	27·76	2·20	27·90

The next experiment was performed similarly, but the saliva was centrifuged.

Results:

Free P before incubation	15·19 mgs.
„ „ after „	15·19 mgs.
Total P	17·84 mgs.

No increase occurred on incubation, and it was thought that the centrifuging must have removed the enzyme.

The experiment was repeated, using the saliva from the same person, but the centrifuging was omitted.

Results:

Free P before incubation	20·82 mgs.
„ „ after „	22·06 mgs.
Total P	23·18 mgs.

In this experiment there is a definite increase.

The following experiments were performed to test the possibility of a hydrolyzing enzyme being present in the desquamated epithelial cells which were thrown down in the centrifuging.

In a 30 cc. sample of saliva free phosphate was estimated at once in 10 cc. (A). The remaining 20 cc. were centrifuged for about 15 minutes. 10 cc. of the clear supernatant saliva were placed in one test tube (B), and the remaining 10 cc. containing the desquamated cells in another (C). After the addition of chloroform both were incubated for 24 hours at 37°C., after which free phosphate was again determined.

Results:

(A)	Free P before incubation	15·61 mgs.
(B)	„ „ after „	15·61 mgs.
(C)	„ „ „ „	16·18 mgs.

Thus there was no increase of free P in the saliva from which the desquamated cells had been removed by centrifuging, whereas that containing these cells showed a small increase.

The next step was to see whether the enzyme in saliva could hydrolyze a solution of sodium glycerophosphate, which is a readily obtainable substance in which the phosphate is organically combined.

Approximately 1 gm. of sodium glycerophosphate was dissolved in a litre of water, and the experiment set up as follows:

A. 10 cc. saliva plus 10 cc. glycerophosphate solution;
B. 10 cc. saliva plus 10 cc. glycerophosphate solution.

Free P was estimated at once in A, while B was incubated for 24 hours at 37°C., and free P then estimated. 10 cc. of trichloracetic acid were added, and the phosphate calculated in 20 cc. of the filtrate.

Results (mgs. P in 20 cc. of the filtrate):

A	1·220 mgs,
B	1·318 mgs.

An increase of 0·098 mgs. occurred. The experiment was repeated, using a stronger solution of glycerophosphate. Approximately 1gm. was dissolved in 250 cc. of distilled water. The experiment was then set up as follows:

A. 10 cc. saliva plus 10 cc. glycerophosphate.
B. 10 cc. saliva (uncentrifuged) plus 10 cc. glycerophosphate.
C. 10 cc. saliva plus 10 cc. water.
D. 10 cc. saliva plus 10 cc. glycerophosphate.
E. 10 cc. boiled saliva plus 10 cc. glycerophosphate.

The free phosphate was estimated at once in A and B, with 10 cc of trichloracetic acid after precipitation of the proteins, and in C, D, and E after incubation for 18 hours at 37°C.

The following figures were obtained:

A	1·500 mgs.
B	1·485 „
C	1·612 „
D	2·174 „
E	1·578 „

The increase in free phosphate due to enzymic hydrolysis of the organic phosphate contained in the saliva itself is given by (C — A) = 0·112 mgs. P. The glycerophosphate itself contained no free phosphate.

The increase due to the action of the saliva on the glycerophosphate is given by (D — A) — (C — A) = 0·562 mg. P.

The control tube, containing boiled saliva and glycerophosphate, showed only a slight increase, 0·093, as compared with the tube containing unboiled saliva and glycerophosphate, 0·562. This experiment, therefore, shows that the enzyme in saliva can hydrolyze sodium glycerophosphate with liberation of phosphate.

The following experiments provide further evidence that the enzyme is contained in the desquamated epithelial cells contained in the saliva.

A 30 cc. sample of saliva was collected. 20 cc. were centrifuged for 25 minutes till the supernatant liquid was clear. The experiment was then set up as follows:

A. 10 cc. saliva (uncentrifuged) plus 10 cc. glycerophosphate.
B. 10 cc. saliva (clear supernatant fluid) plus 10 cc. glycerophosphate.
C. 10 cc. saliva (containing the desquamated cells) plus 10 cc. glycerophosphate.

Free phosphate was estimated in A at once, and in B and C after incubation with chloroform at 37°C. for 24 hours.

Results (mgs. P in 20 cc. filtrate):

A 1·281 mg.
B 1·388 „
C 2·116 „

These results again show a large hydrolysis in the presence of desquamated cells.

It was next thought advisable to find out whether the desquamated cells could exert this hydrolytic property apart from saliva. In other words, to see whether a co-enzyme is present in the saliva.

Twenty cc. of saliva were centrifuged till all the cells had been thrown down. The supernatant saliva was poured off, and the deposit well mixed with normal saline and again centrifuged. This washing was repeated twice. Finally, after pouring off the last lot of saline, the volume was made up to 20 cc. with distilled water, and the cells were thoroughly mixed with it.

The experiment was set up as follows:

A. 10 cc. glycerophosphate plus 10 cc. water.
B. 10 cc. glycerophosphate plus 10 cc. cell suspension.
C. 10 cc. glycerophosphate plus 10 cc. cell suspension.

In C the cell suspension was heated to 80°C. for four minutes.

The tubes were incubated for 24 hours at 37°C. It was then necessary to see whether any phosphate had been set free in inorganic form. As a standard for comparison 10 cc. of the glycerophosphate solution was used. This was

placed in a 100 cc. volumetric flask and the reagents added. An extremely pale blue colour developed. For comparison, the tubes A, B, and C were filtered, and the filtrates similarly transferred to 100 cc. flasks. The reagents were added and the colour allowed to develop freely.

Results:

A. No increase in colour.
B. Definite increase.
C. Very slight increase.

This experiment shows conclusively that the enzyme is contained in the desquamated epithelial cells, and that no co-enzyme is necessary. The slight increase noted in C is probably due to insufficient heating of the cells.

The previous experiment was repeated, but the cell suspension in tube C was boiled for 6 minutes. After incubation the increase in tube B was estimated quantitatively in 15 cc. of the filtrates, a standard containing 0·2 mg. P being used.

Results:

A. Nil.
B. 4·34 mgs. per 100 cc.
C. Trace only.

This experiment agrees with the foregoing one, and in addition shows that the enzyme is destroyed by boiling for 6 minutes.

THE INFLUENCE OF pH ON THE HYDROLYTIC POWER OF THE ENZYME.

In this series of experiments the optimum pH of the enzyme was determined. As a criterion of its action, the amount of inorganic phosphate set free from sodium glycerophosphate was taken. The hydrogen ion concentration was adjusted by means of a Hellige comparator.

Fifty cc. of saliva were collected, and this was divided into five portions of 10 cc., and to each was added 10 cc. sodium glycerophosphate solution. One tube was set apart for the pH estimations.

In tube D the free phosphate was estimated at once. To the others was added a sufficient amount of alkali or acid, according to the pH required in the experiment. 0·2 cc. chloroform was added to each tube, and then enough distilled water to make the total volume added up to 3 cc. After incubation for exactly 8 hours at 37°C., 7 cc. 10% trichloracetic acid were added and free phosphate estimated.

Results:

Tube.	pH.	Inorg. P after incubation.	Inorg. P before incubation.	Increase.
A	5·5	1·786 mg.	1·235 mg.	0·551 mg.
B	8·1	1·575 „	1·235 „	0·340 „
C	9·4	1·274 „	1·235 „	0·029 „

This experiment shows the greatest increase in the most acid tube. The experiment was repeated with different hydrogen ion concentrations.

Results:

Tube.	pH.	Inorg. P after incubation.	Inorg. P before incubation.	Increase.
A	6·9	1·122 mg.	0·990 mg.	0·132 mg.
B	7·9	1·059 „	0·990 „	0·069 „
C	9·4	0·951 „	0·990 „	—

In this experiment the largest increase is also on the acid side, while at pH 9·4 no hydrolysis whatever occurred.

The last experiment was repeated, using saliva from a different person.

Results:

Tube.	pH.	Inorg. P after incubation.	Inorg. P before incubation.	Increase.
A	7·0	1·053 mg.	0·823 mg.	0·230 mg.
B	7·9	1·036 „	0·823 „	0·213 „
C	9·4	0·893 „	0·823 „	0·070 „

Although larger increases occurred in this case, the relative increases at different pH values were the same as in the last experiment. The greater increases noted are probably due to the larger quantity of enzyme present in the particular saliva used.

In the next experiments, in order to determine the optimum pH, tubes of higher acidity were set up.

Results:

pH	Inorg. P after incubation.	Inorg. P before incubation.	Increase.
5·0	1·370 mg.	0·897 mg.	0·473 mg.
6·0	1·266 „	0·897 „	0·369 „
7·0	1·149 „	0·897 „	0·252 „
4·5	1·470 „	1·197 „	0·273 „
5·0	1·527 „	1·197 „	0·330 „
5·5	1·575 „	1·197 „	0·378 „

These experiments show, therefore, that the optimum reaction for the phosphatase present in the desquamated epithelial cells is in the region of pH 5·5, and the enzyme can thus exert its influence on calculus deposition in a medium which may be distinctly on the acid side of neutrality.

Nature of the Organic Phosphate in the Saliva.

An attempt to isolate the organic phosphate had been originally intended, but the attempt was abandoned when it was realized that it occurred in such small amount. Nevertheless, a considerable amount of evidence has been obtained to show that the compound is in the nature of a phosphoric ester probably of the hexosephosphoric group. Adamson (1) showed that an increase of free phosphate in saliva, accompanied by an increase in copper-reducing power, occurred on hydrolysis with 0·1 N acid. The present writer

has confirmed this observation, and in addition has shown that the same changes may be brought about by enzyme action.

McLean's blood sugar method was used for estimating the sugar in saliva, using 10 cc. saliva and 10 cc. acid sodium sulphate. These were heated together and 5 cc. dialyzed iron added. In this way the subsequent filtration was rapid, and a clear filtrate obtained. 15 cc. of the filtrate were used for the estimation. It is important that the saliva should be entirely free from starch, as the action of ptyalin would rapidly convert it into sugar. The teeth must therefore be carefully brushed and the mouth well rinsed with water before the saliva is collected.

Adamson's experiment, in which the protein-free filtrate was hydrolyzed by boiling with acid, was repeated.

0·15 cc. N hydrochloric acid was used instead of sulphuric, and hydrolysis was carried out for one hour on a boiling water bath.

Results:

Before boiling with acid=0·0012% sugar as glucose
After „ „ „ =0·0043% „ „ „
Increase=0·0031% „ „ „

This result is in agreement with values obtained by Adamson.

A similar experiment was then carried out, in which the free phosphate was estimated instead of the sugar. 20 cc. of saliva were collected, and proteins precipitated as usual. In the filtrate free and total phosphates were estimated. 30 cc. of the same filtrate were heated on a boiling water bath with 10 cc. of normal hydrochloric acid, under a reflux condenser, for 6 hours.

Results (in mgs. P per 100 cc. saliva):

Inorganic P before boiling with acid=19·72 mgs.
„ „ after „ „ „ =20·80 „
Increase= 1·08 „
Total phosphate .. .. .. =21·17 „
Original combined phosphate .. = 1·45 „
Amount hydrolyzed .. .. =74·5%

This experiment confirms the result of Adamson, who found that there is a combined phosphate in saliva which is hydrolyzable by dilute acid, giving an increase in free phosphate. As previously shown, the action of an enzyme, present in saliva, can bring about the same result, and, as is shown in the following experiments, an increase in reducing power also occurs.

Twenty cc. saliva were collected. The sugar in one half was estimated at once, and the other half was incubated for 22 hours at 37°C. in presence of chloroform.

Results:

Before incubation=Nil
After „ =0·0098% sugar
Increase=0·0098% „

Many of the samples of saliva examined showed no trace of reducing power, and in none of them was the amount of sugar appreciable. This experiment shows a much larger increase than that obtained by acid hydrolysis.

To prove that the increase of sugar is due to enzyme action, the saliva was boiled before incubating, with the following results:

30 cc. of saliva was divided into three 10 cc. portions.

A. Estimated free sugar at once.
B. Boiled for 1 minute, then incubated with chloroform for 24 hours at 37°C. Sugar then estimated.
C. Incubated for 24 hours with chloroform, but without previously boiling.

This experiment was repeated twice.

Results:

	1	2	3
A.	Nil	0·0013%	0·0017%
B.	0·0011%	0·0018%	0·002%
C.	0·0038%	0·0063%	0·0113%

In each case there was a marked increase of reducing power, which increase was prevented by boiling for 1 minute beforehand.

Several possibilities which would lead to an increase of sugar on incubation had to be considered.

1. Contamination of the saliva with starch, which might be hydrolyzed to sugar by the ptyalin. Care was taken, however, in cleansing the mouth thoroughly before collecting the saliva. Any sugar from such a source would be estimated in the control, as the action of ptyalin is very rapid.

2. Possibility of hydrolysis of the gluco-protein mucin. Bacterial action was prevented by the addition of chloroform. In addition, an increase of sugar was obtained by boiling protein-free filtrates with acid.

3. Enzymic hydrolysis of a sugar-containing organic phosphate by means of the phosphatase contained in the desquamated cells. Although incubating saliva, from which the epithelial debris had been removed, was not tried, it is almost certain that a result similar to that found with the phosphates would have been obtained. It has been assumed, therefore, that the increase in sugar is due to the action of the same enzyme that brings about an increase in free phosphate when saliva is incubated.

The results of these experiments, therefore, show that, when a protein-free filtrate of saliva is boiled with dilute acid, an increase in free phosphate occurs, which is accompanied by an increase in reducing sugar, and also that similar changes are brought about when saliva is incubated, by means of an enzyme contained in the desquamated epithelial cells. These changes point to

the fact that the organic phosphate in saliva is of the nature of a phosphoric ester, probably of the hexosephosphoric group.

Enzyme Content of Saliva of Different Persons.

It is a commonly recognized fact that dental calculus is always associated with gingivitis, but the usual conception is that the calculus precedes and is the cause of the gingivitis. The writer, however, hopes to show that this is not the case, and that inflammatory changes in the gingivae can be one of the most important factors in originating the deposition of calculus. It has been shown that the epithelial cells, which are normally cast off from the surface of the mucous membrane, contain a phosphatase, i.e., an enzyme which converts organic phosphate into inorganic form. This shedding of the surface layers of the stratified epithelium is a normal process, but in conditions in which the mucous membrane is inflamed, and perhaps ulcerating, this desquamation is increased, and the saliva will naturally contain more of the cast-off epithelial cells. It is logical to suppose, therefore, that in patients exhibiting inflammatory changes of the gingivae and oral mucous membrane generally there will be a larger amount of phosphatase present in the saliva. The following work was carried out to show experimentally whether or not this supposition is correct.

Patients showing definite inflammation of the gums were obtained, and the amount of calculus was noted. The phosphatase content of the saliva was estimated by finding the amount of sodium glycerophosphate hydrolyzed under the same conditions by different salivas. 20 cc. of saliva were collected from each patient. Inorganic phosphate was estimated at once. The experiment was then set up as follows: 10 cc. saliva + 10 cc. glycerophos. solution+ 0·2 cc. chloroform. The tube was then incubated for exactly 8 hours at 38°C. Any increase in free phosphate after incubation will be a measure of enzyme action, the increase being partly due to hydrolysis of the organic phosphate of the saliva, but mostly due to hydrolysis of the glycerophosphate. By incubating for only 8 hours, and having an excess of substrate for the enzyme to act upon, the increases noted should be proportional to the amount of the enzyme present.

Results (expressed in mgs. P contained in the 20 cc. of filtrate taken for estimation):

Case.	Condition of Gums.	Calculus.	Hygiene.	Free Phosphate. Before Incubation.	After Incubation.	Increase.
1.	Slightly inflamed	Around lower incisors	Poor	0·920	1·220	0·300
2.	Just over acute infection	Around lower incisors	Neglected	0·976	1·298	0·322
3.	Bluish and unhealthy; poor tone; chronic inflammation	Around lower incisors and reforms rapidly	Cleans teeth well	1·379	1·942	0·563

Case.	Condition of Gums.	Calculus.	Hygiene.	Free Phosphate. Before Incubation.	After Incubation.	Increase.
4.	Fairly healthy	Small amount at lingual aspect of lower incisors	Cleans teeth well	0·866	1·026	0·160
5.	Healthy	Large deposit at lingual aspect of lower incisors, also on occlusal of lowers: reforms rapidly	Cleans teeth well	1·176	1·538	0·362
6.	Grossly inflamed generally	Present in great amount all over the mouth; never been scaled	Quite neglected	0·823	1·036	0·213
7.	Healthy	No deposit nor tendency	Good	1·235	1·575	0·340
8.	Gums generally inflamed	Around upper molars and lower incisors	Quite neglected	1·006	1·154	0·148
9.	Not very inflamed	Serumal calculus generally present	Cleans teeth well	0·961	1·127	0·166
10.	Generally inflamed	Not much calculus, but every tooth loose	Quite neglected	1·230	1·515	0·285

Although the results obtained do not allow any definite conclusions to be drawn, there was a marked increase in free phosphate in every case, indicating the presence of the enzyme. In attempting to estimate the quantity of enzyme present in saliva several factors tending to influence the result must be considered. In the first place, the portion of saliva that is collected first will contain more desquamated cells than the portion collected towards the end; so that if in any particular case more than the intended 20 cc. were collected there would be a greater dilution of the enzyme. Thus in case 6 in the above table, in which a high result was expected, a small increase occurred, owing to the fact that there was an abnormal flow of saliva and 50 cc. were collected before the amount was observed.

In addition, if the teeth and gums have been brushed a short time before the collection of the specimen, a low content of cells would result. The chewing of rough food would exert a similar influence. Unfortunately these factors were not controlled in the above estimations, so that, although the figures obtained do not show what was intended, they cannot be taken as negative evidence.

The writer, to show whether brushing of the gums results in a decrease in enzyme content, performed the following experiment:

Two 20 cc. samples of saliva were collected from a patient having normal gums and oral mucous membrane. The first sample was collected after a breakfast consisting of very soft food, and brushing of the teeth and gums was omitted. The second sample was then collected after vigorously brushing the gums and other parts of the mouth. Free phosphate was estimated in both samples, and 10 cc. of each were then incubated for 8 hours with 10 cc. of sodium glycerophosphate solution.

After incubation the free phosphate was again estimated.
Results (in mgs. P in 20 cc. of filtrate).

	Without brushing.	After brushing.
Before incubation	1·270 mgs.	1·127 mgs.
After „	1·875 mgs.	1·376 mgs.
Increase	0·605 mgs.	0·249 mgs.

The results show that nearly two and a half times more phosphorus was set free by the action of the enzyme in the first sample of saliva than by the enzyme contained in the second sample. This was directly due to the fact that most of the desquamating cells were removed by the vigorous brushing received before collection of the second sample of saliva. This fact adds still further weight to the importance of the use of the toothbrush in preventing calculus deposit.

Calcification of the Epithelium of the Gums.

Robison produced a deposit of calcium phosphate in rachitic bones by immersing them in solutions of phosphoric esters of calcium under suitable conditions. This deposition was due to the hydrolytic action of the phosphatase contained in the bones on the organic phosphate. It is now recognized that a similar reaction plays a very important part in the normal process of ossification of bones.

Now it has been shown that the epithelial cells of the oral mucous membrane contain a similar phosphatase, and also that blood serum and saliva contain organic phosphates capable of being hydrolyzed by the enzyme, with a resulting liberation of free phosphate ions.

It is certain that a deposition of dental calculus in the gingival trough can result from a liberation of the phosphatase from the desquamated cells and its action on the organic phosphates of the saliva and exuded serum.

It does not seem illogical, therefore, to suppose that under certain conditions an interaction between phosphatase and organic phosphate can take place actually within the epithelial cells before they are desquamated, resulting in an increase of PO_4 ions; if the fluids bathing the cells are saturated in respect to calcium phosphate, then such an increase will result in the deposition of the solid salt within the cells. It is improbable that any such action can take place within normal cells, but if any damage has occurred it is possible that, owing to a disturbance of the enzymic balance within the cells, the phosphatase can exert its action.

It is a pathological axiom also that degenerated tissues are specially liable to become calcified, and the present writer is of the opinion that the action of phosphatases, which are present in many tissues, will eventually prove to be one of the main factors in many cases of pathological calcification.

Retrogressive changes in the epithelium of the gum may be brought about

not only by local causes, but also by causes affecting the general health of the patient. It has been shown by Howe and Wolbach (17) that by feeding animals on diets deficient in vitamin a degeneration of epithelium could be produced. This is suggestive from the point of view of calculus formation and its relation to tissue changes in the mouth. There is the possibility of deficient diet leading to degenerative changes in the epithelium of the gums resulting in an increased desquamation of cells with their contained phosphatase, or actual calcification of the cells *in situ*. Adamson is of the opinion that such calcification can occur, and states that "in some instances the epithelium lining the gingival trough appears to undergo pathological calcification." This statement was based on the examination of post-mortem sections which certainly do seem to show the condition described.

The following experiments were carried out in the hope of proving that such a process actually occurs. As a preliminary experiment it was decided to find out whether a deposit of calcium phosphate, such as Robison obtained in bones, could be produced similarly in the epithelium of the gums.

Pieces of tissue were removed from the free gum margin after tooth extraction. In each experiment the tissue removed was cut into two halves. One half was placed in a filtered saturated solution of calcium glycerophosphate (a sparingly soluble substance), and the other in normal saline to act as a control. Both were incubated for 24 hours at 37°C.

The specimens were then fixed in 5% formol saline, and embedded in paraffin in the usual way. After washing the sections thoroughly in distilled water, they were treated with 1·5% $AgNO_3$ for 30 minutes in sunlight. After washing thoroughly again the nuclei were counterstained with safranin, or light staining with haematoxylin and eosin was carried out.

In every case in which the gum tissue had been in calcium glycerophosphate there was a dense black band in the more superficial layers of the epithelium easily visible under the low power of the microscope. On closer examination the black areas were seen to be made up of numerous small black granules coalescing in the most deeply stained parts. In many cases the surface epithelium was stained completely black and often partly detached from the surface.

The subepithelial connective tissue in some cases showed a slight deposit in the papillae, but generally the deposit was limited to the epithelium.

As further controls solutions of sodium phosphate and calcium chloride were used in different experiments to make sure that the staining was not due to mere adsorption of the glycerophosphate. In the controls a dense deposit never occurred, although with a very high power a few extremely fine black granules were sometimes visible. The amount of staining with silver nitrate in the controls, however, was never appreciable, and the granules were scattered irregularly throughout the epithelium.

Photomicrographs of the deposit in the epithelium obtained by immersion in calcium glycerophosphate are shown in Plate 1, figures 3 and 4.

The method of staining with silver nitrate (Von Kossa's method) is a definite histo-chemical test for the presence of calcium phosphate, and depends on the formation of an insoluble silver phosphate, which is reduced to black silver oxide by the action of the sunlight.

It may therefore be concluded that a deposition of calcium phosphate in the epithelium of the gums has been produced, and that the deposition is due to an interaction between the phosphatase contained in the epithelial cells and the calcium glycerophosphate present in the experiment.

Having produced such a deposit *in vitro*, the next experiments were performed in the hope of showing a similar deposition of calcium phosphate in the epithelial cells during life.

Pieces of gum tissue were removed from the mouths of patients who presented themselves for tooth extraction. The specimens obtained were lying beneath deposits of calculus, and showed inflammatory and degenerative changes to a varying degree. On the other hand, pieces of normal epithelium were obtained to serve as a means of comparison.

The specimens were well washed in saline, fixed in formalin, and embedded in the usual manner. Staining with silver nitrate was carried out as in the previous experiment. Ten specimens were thus examined, but it was not possible to show any relation between a heavy staining of the epithelium with silver nitrate and a deposit of calculus occurring on its external surface.

It was felt, however, that the technique could be improved, and in the next series it was modified as follows: After fixation the tissue was washed in several changes of distilled water for 48 hours. It was then placed in 2% silver nitrate for 2 hours in the dark. Washing for 24 hours and dehydration were similarly carried out in the dark, after which clearing and embedding were done in the usual manner. The blocks were sectioned, and staining with safranin or haematoxylin and eosin was carried out as before. In the last series some irregular results were obtained, probably through silver being deposited on the slide independently of any particular substance present in the tissue, but in this method only silver that has actually combined with, or been reduced by, compounds in the tissue will be observed in the section.

The results in this series of another ten cases were again negative. Each section, whether from normal or degenerated epithelium obtained from beneath calculus deposit, showed an approximately equal amount of staining with silver nitrate. This was evidenced by small black granules scattered irregularly throughout the different zones of the epithelium. The granules most frequently were arranged in the form of a ring just inside the nuclei of the epithelial cells. It was thought quite obvious that the granules here observed could not be due to calcium phosphate deposited pathologically in the epithelium.

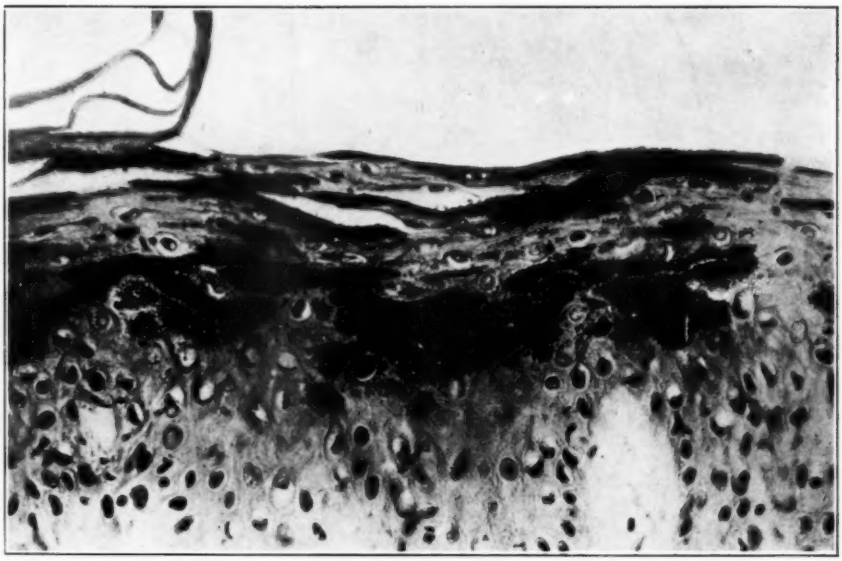


Fig. 3. Epithelium of the gums after immersion in calcium glycerophosphate. Stained with Ag NO_3 and safranin. Note granules in the superficial layers coalescing into deeply staining black masses.

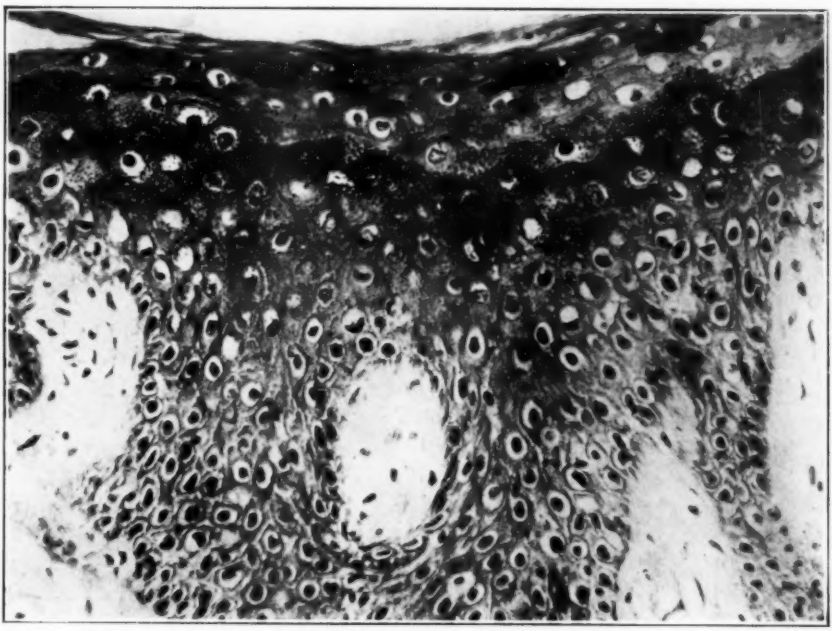


Fig. 4. Same as above, but stained with haemotoxylin and eosin after Ag NO_3. Note the granular nature of the deposit.

PLATE 1.

Although a deposit of calcium phosphate has been produced experimentally in the epithelium of the gums, it has not been possible in the above experiments to demonstrate its presence in epithelium taken directly from the mouth.

SUMMARY.

In the foregoing pages the writer has set forth the ideas and experimental results of other workers, and where possible has performed experimental work in the hope of shedding new light on what is a most obscure subject.

The different theories which have been held responsible for calculus formation, and which have been discussed separately in this paper, are briefly summarized as follows:

1. Loss of carbon dioxide.
2. Physico-chemical theory.
3. Diet as related to:
    (a) The pH of saliva;
    (b) The calcium content of saliva.
4. The rôle of organisms.
5. Enzyme theory.

It is quite certain that no single factor is wholly responsible for calculus formation, and that many different factors exert their effects at the same time. The theory of enzyme action, as advanced by Adamson, is the most recent view put forward, and the greater part of the experimental work in this paper has been devoted to this aspect of the subject.

The conclusions arrived at by a consideration of the experimental results obtained are set out hereunder.

CONCLUSIONS.

1. Loss of CO_2 from saliva is of slight importance as a cause of dental calculus deposit.

2. The pH of saliva is not influenced by the ingestion of different types of diet, nor does it correspond with changes of urinary pH, which can be readily produced by such alteration of the diet.

3. The pH curve for saliva takes approximately the same course each day, irrespective of the diet and the urinary pH.

4. It is probable that the pH of saliva does not show large enough variation from the normal to exert any marked influence on calculus.

5. A method for the estimation of phosphoric acid in saliva is described, and values of organic and inorganic phosphates are given.

6. There is no observable relation between the amount of organic phosphates present in saliva and a tendency to calculus deposit.

7. The presence of a phosphatase in saliva has been confirmed. This enzyme is contained in the desquamated epithelial cells, which are normal constituents of the saliva.

8. This salivary phosphatase brings about an increase in free phosphate by its action on the organic phosphate compound contained in the saliva. It exerts a similar hydrolytic action on sodium glycerophosphate.

9. The optimum reaction for the salivary phosphatase is in the region of pH 5·5.

10. The organic phosphate in the saliva is probably of the nature of a phosphoric acid ester.

11. The phosphatase content of the saliva of different persons depends largely upon the conditions of the gums and oral mucous membrane.

12. The use of the toothbrush diminishes the amount of phosphatase in saliva, and shows the importance of brushing the gums as a prophylactic measure against calculus formation.

13. A deposition of calcium phosphate in the epithelium of the gums has been produced *in vitro*.

14. Attempts to show that such a deposit occurs in epithelium taken directly from the mouth have not been successful.

In conclusion, the author's thanks are due to Associate Professor W. J. Young, in whose laboratory the work was carried out, for advice and help during the investigation, and to Professor F. C. Wilkinson for his encouragement and much valuable criticism.

REFERENCES.

1. Adamson, K. T.: Austr. Journ. Exper. Biol. and Med. Sci., 6 (1929), p. 215.
2. Adamson, K. T.: Austr. Journ. Dent. (Aug., 1929), p. 255.
3. Badanes, B. B.: Dental Cosmos (Sept., 1929), p. 909.
4. Baehr, G.: Bull. New York Acad. Med. (1927), p. 419.
5. Black: "Special Dental Pathology" (1915), p. 84.
6. Bloomfield, A. L. and Huck, J. G.: Johns Hopkins Bull. (1920).
7. Bulleid, A.: Brit. Dent. Journ. (March, 1925), p. 289.
8. Bunzell, H. H.: Colgate & Co.'s Bull., No. 1 (1923).
9. Burchard, H.: Dental Cosmos (1895), p. 821.
10. Burchard, H. and Inglis: "Dental Pathology" (1927), chap. 19.
11. Carlson, V. R. and McKinstry, M. L.: Dental Cosmos (1924), p. 840.
12. Clark, G. W.: Dental Cosmos (1925), p. 500.
13. Clark, G. W. and Levine: Amer. Journ. Physiol. (1927).
14. Fiske, C. H. and Subbarow, Y.: Journ. Biol. Chem., 56 (1925), p. 375.

15. Goodrich, H. and Mosely, M.: Journ. Roy. Micros. Soc. (1916), p. 513.
16. Henderson, M. and Millet, J. A.: Journ. Biol. Chem., 75 (1927), p. 559.
17. Howe, P. and Wolbach, J.: Journ. Exper. Med., 42 (1925), p. 753.
18. Hulin, C.: Compt. Rend. Soc. Biol., 98 (1928), p. 185.
19. Kaye, G.: Austr. Journ. Exper. Biol. and Med. Sci., 6 (1929), p. 419.
20. Kelsey, C. J.: Brit. Dent. Journ. (1910), p. 810.
21. Kirk, E. C.: American Text-book of Operative Dentistry (1911).
22. Kirk, E. C.: "Mouth Hygiene," p. 160.
23. McClelland, J. R.: Amer. Journ. Physiol., 63 (1922), p. 127.
24. McClelland, J. R.: Dental Cosmos (1924), p. 425.
25. Martland, M. and Robison, R.: Biochem. Journ., 20 (1926), p. 852.
26. Naeslund, C.: Dental Cosmos (1926), p. 1137.
27. Pattison, C. L.: Brit. Med. Journ. (July 26, 1926).
28. Payne: Brit. Dental Journ. (1924), p. 1641.
29. Pickerill, H. P.: "Prevention of Dental Caries," chap. 12.
30. Prinz, H.: Dental Cosmos (1921), p. 504.
31. Robison, R.: Biochem. Journ. (1923), p. 286.
32. Starr, H. E.: Journ. Bio. Chem., 54 (1922), p. 43.

FURTHER RESULTS IN BLOOD GROUPING CENTRAL AUSTRALIAN ABORIGINES

by

J. BURTON CLELAND

(Submitted for publication 15th May, 1930.)

In August, 1929, a small expedition, under the auspices of the Board of Anthropology of the University of Adelaide, and partly financed by the Rockefeller Foundation through the Australian National Research Council, paid a visit to Hermannsburg Mission Station in Central Australia. Hermannsburg is situated about 80 miles west and a little south of Alice Springs, which is the administrative centre of the Territorial Division of Central Australia. Alice Springs is approximately midway between the eastern and western boundaries of the Territory and about on the Tropic of Capricorn. Its inhabitants are thus obviously in the geographical centre of Australia.

One of the objects included in the programme was the blood-grouping of a further number of Central Australian aboriginals. Seventy-one individuals belonging to the Arunta and Loritcha were examined, comprising 70 pure-bloods and 1 half-caste accidentally included. The red cells of these, tested against the key sera II (b) and III (a) of European origin, showed that 24 full-bloods and the 1 half-caste belonged to Group O, and 46 full-bloods to Group A. These results were perfectly clear cut, and in no instance was there any ambiguity.

As a check upon these results, the tests were inverted, European red cells of these same Groups II (A) and III (B) being tested against the sera of the aboriginals. With the exception of certain anomalous results to be discussed later, this reversing confirmed completely the original determinations.

With the object of testing for anomalies, as for instance certain sub-groups that have been recorded in other races, as many aboriginal red cells were tested against aboriginal sera as circumstances permitted. Red cells were thus tested against sera of their own group, as well as against sera of the other group met with in the aboriginals. A total of 293 cross-tests were thus performed, which revealed the presence occasionally of unexpected reactions, indicating the presence of sub-groups. These reactions will also be dealt with later.

No difficulty was experienced in any way in carrying out the tests. The work was greatly facilitated by the authorities at the Mission, to whom we would like to express our indebtedness for the unqualified success of our visit.

Pastor Albrecht and Mr. Heinrich, who had the full confidence of the natives, explained to them the objects of our visit, and they readily fell in with all our wishes. By a piece of good fortune, our numbers (there were eight of us) coincided with the number of divisions in the Arunta. One of the natives made this discovery, with the result that we were immediately allocated in all seriousness each to one division and accepted as full members of the tribe. A plenitude of tribal relatives sprang up—parents, uncles and aunts, brothers and sisters were in embarrassing profusion, and most cordial relationships existed, facilitating greatly the enquiries on which we were engaged. This attitude may be emphasized as indicating part of the psychology of the Australian aboriginal. During the carrying out of nearly three hundred blood-groupings on these natives we have never met with any opposition or any suspicions as to our intentions, in striking contrast to the experiences of others with some primitive peoples.

We had no difficulty in obtaining blood by puncturing the lobe of the ear with a triangular needle, and the tag of cotton wool applied to stop the bleeding, contrasting with the dark hue of the skin, gave temporary distinction to the fortunate possessor. Professor T. Harvey Johnston (who kindly assisted me) and myself soon gained the soubriquet of "the butchers" from the surrounding aborigines, and our mild form of operation was designated, in cattle terms, as "ear-marking." It soon became the fashion to be ear-marked, and those who had it not eagerly desired it. As we were not dealing with half-castes, some of these were bitterly disappointed at our refusal to bleed them.

The work carried out at Hermannsburg, and the results, will be considered under the following heads:

(I) The methods employed.

(II) The significance to be attached to these further data, showing that the aboriginals of Central and South Australia possess only two blood-groups, viz., A and O, and a discussion of the distribution of these two groups in this area.

(III) The anomalous results, indicating the presence of sub-groups, found occasionally when testing aboriginal sera against European red cells as well as against aboriginal red cells.

I. THE METHODS EMPLOYED.

These were essentially the same as those described in our last communication (3) on this subject. The blood was drawn from the lobe of the ear by puncturing with a triangular needle. A moiety of this was received straight away in the red cell pipette of a haemocytometer, and diluted with normal saline solution to 1 in 100, as recommended by Dyke (5). This diluted red cell suspension was only used on the day on which it was prepared. Red cells more

than eight hours old were never employed. This precaution was taken to preclude the possibility of any anomalies met with being due to the age of the red cells. As soon as the requisite moiety of blood for the red cells had been taken, a small tube of blood was collected, allowed to clot, and the serum then separated. The sera thus obtained were used not only on the day when they were taken, but also on subsequent days, and samples of most were brought back to Adelaide, and there tested against the red cells of Group III (B) belonging to the donor of our type serum III (a).

As was the case in our last two expeditions, my own serum was used as the source of Key Serum II (b), and my own red cells were tested by reversing with the serum of each aborigine tested. Dr. Hackett, who supplied Key Serum III (a) on the two previous occasions, having left for Europe, Dr. Pitcher, to whom my thanks are specially due, supplied Key Serum III (a) instead, and on return to Adelaide sera from most of the natives tested were available for reaction with his red cells. As we had with us Mr. Tindale, belonging to Group B, we used his red cells also, as we did on a previous occasion, for testing in the field. The work was done by daylight in the schoolroom at Hermannsburg, at room temperature under mild spring conditions, the days being quite warm, but the nights occasionally chilly. The reactions were read with a pocket lens, supplemented when necessary by the low power of the compound microscope, and the results were checked up to half an hour after setting up. Tests yielding anomalous results were sometimes repeated several times, always with the same results.

II. THE PRESENCE OF ONLY GROUPS A AND O IN CENTRAL AND SOUTH AUSTRALIAN ABORIGINALS, AND THEIR RELATIVE GEOGRAPHICAL DISTRIBUTION.

The results at Hermannsburg showed that, with the Key Sera II (b) and III (a), 46 full-bloods belonged to Group A and 24 to Group O. There was a complete absence of the agglutinable substance B, either in the shape of Group B or of Group AB. We (1, 2, 3) have now tested 296 full-blood aboriginals belonging to Central or South Australia, and of these 173 belonged to Group A and 123 to Group O, i.e., a little less than 59% contained the agglutinable substance A only, and rather more than 41% neither A nor B. We have already (11) discussed the significance of this absence of B. It may signify that the Australian aboriginal broke away from a stock in which the agglutinable substance B had not yet appeared in the red cells, either by mutation or by admixture with another stock in which B had developed. On the other hand, the absence of B may be explained by the population with which we are concerned being descended from a small family group of castaways in whose make-up it chanced that B was not present.

Do these results bear on the question as to the purity of the Australian aboriginal? It seems to us that they do. There is no doubt that within comparatively recent times there has been a certain amount of intermixture with Malay blood along the north-western and northern coasts of Australia, and such should have introduced there the agglutinable substance B. It is quite likely that on the west coast early European castaways have left descendants who might again have introduced B. It is probable that Papuans and islanders from Torres Straits have intermixed slightly with the natives of the north-east, and the large kanaka population at one time in Queensland doubtless left its mark. All these intermixtures must be considered as having occurred within the last few centuries, and most of them quite recently. The Australian aboriginal, on the other hand, with the dingo, has been here for many thousands of years, some evidence suggesting his presence when the extinct volcanoes of Mount Gambier and the Warrnambool district were still active. Supposing then the Australian aboriginal to have originally been a pure race (and we believe such was the case), and that it was composed of two blood-groups only, namely A and O, contamination has undoubtedly occurred recently along the north and north-west coasts, and probably along the north-east coast, but such contamination of extra-European origin has not had time to permeate into the interior or to extend to the south. We would expect this foreign element (though diluted so that individuals contaminated by it might be indistinguishable from full-bloods and pass for such) to manifest its presence by the introduction in some instances of the agglutinable substance B into the make-up of the tribe affected. Thus B would appear to some extent in the descendants of, say, a Malay trepang-fisher who was visiting the coast of Australia at the time Flinders surveyed these coasts 130 years ago, if he belonged to Groups B or AB and had offspring by an aboriginal woman. After five or six generations without further admixture of foreign blood, the general appearance of his descendants might not differ from pure-bloods, though the agglutinable substance B might be retained. The researches of Tebbutt (9) and of Lee (7) do show that in Queensland there is a small percentage of natives, believed to be full-bloods, who contain B in their make-up, and Tebbutt and McConnell (10) have even found one in New South Wales. Thus Tebbutt found 5·76% of Group B and 1·05% of Group AB, and Lee 6·4% and 1·6% respectively. We would be inclined to attribute the presence of B to such a recent introduction rather than as supporting the view that in the far-back ages two separate groups of immigrants populated Australia, one entering from the north-west (and possessing only A in its make-up) and the other entering from the north-east (and possessing some B as well as A). We do think that it is quite likely, however, that after reaching Australia somewhere along the north coast, the immigrants (a group of relatives probably) divided eventually into two hordes, one of

which tended to pass down the west coast, and the other near the east coast, each finding along the coastline food supplies more to its liking than in the arid interior. We would not be surprised if, during such collateral descent towards the south coast, one of the hordes developed a greater proportion of A than of O in its make-up, the other the reverse. Being far separated from intermixture for many generations by the dry interior, one horde as it descended would tend to develop or to drop certain customs and habits, the other horde other customs or habits. Finally, when lack of food-supply for the more coastal population led to the peopling of Central Australia, or when the two hordes met circumferentially, their peoples would be again in close association, though now separated by cultural habits as well as possibly by some change in the relative proportions of A and O. Is there any evidence of the latter in the data at present at our disposal? The results we now record, added to our previous investigations (1, 2, 3), suggest that there may possibly be in blood-grouping some support to the view that two hordes, both possessing only A and O, may be in juxtaposition in Central and South Australia. These various results will now be considered.

All the results we have so far obtained may be tabulated in the following order:

	A.	O.
Koonibba and Penong (near the Great Australian Bight) ..	41	19
Tarcoola (natives along the East-West Line from Tarcoola to Ooldea, and probably for many miles north thereof) ..	36	19
Hermannsburg (western Central Australia, Luritcha and Arunta)	46	24
N.E. of South Australia (mostly Cooper natives at Innamincka, Cordillo natives in the N.E. corner)	16	12
Oodnadatta (Arunta)	8	8
Alice Springs (Arunta)	19	22
Point Pearce (in Yorke Peninsula, but natives from various southern sources)	4	4
Point Macleay (natives of Lake Alexandrina, at the mouth of the Murray)	3	13
Adelaide Hospital (one an Arunta)	0	2
Totals	173	123
		296

Of the grand total, the proportion of A to O is a little less than 3 to 2. In the various localities, however, the proportions vary as follows:

Koonibba, Tarcoola, and Hermannsburg, A : O :: 2 : 1 nearly (a little more O in Tarcoola and Hermannsburg[1] natives).

Alice Springs and Oodnadatta (Arunta), A : O :: 1 : 1 nearly (O in excess).

N.E. of South Australia, A : O :: 1 : 1 nearly (A in excess).

Point Macleay, A : O :: 1 : 4 nearly.

It would thus seem that the western natives of South and Central Australia, from the Great Australian Bight to the far north, have a preponderance of nearly 2 to 1 of A over O in the red cells. The Arunta natives at Alice Springs and Oodnadatta, more to the east of the first group, and the natives of the north-east of South Australia, have nearly equal proportions of A and O. Natives at the mouth of the Murray, a gateway for descent by natives coming down from north-east Australia, show a decided preponderance of O, though the numbers tested are too small for stress to be laid on this.

These results suggest that the native horde descending from the north by the west coast of Australia, during their progress developed a greater preponderance of A over O in their blood make-up, whilst the other horde, coming down by an eastern route, showed more or less an equality of A and O, or possibly possessed an excess of O. The two hordes are now in contact in South and Central Australia, but intermixing has not yet produced uniformity in the proportions of A and O throughout these geographical areas, either because the propinquity is comparatively recent, or because cultural, linguistic, and other barriers have maintained relative purity of blood in the two hordes, even though they are in contact. It is of interest to know that these views, based on the blood-grouping, harmonize with inferences drawn from cultural and other aspects. Mr. N. B. Tindale, Ethnologist at the South Australian Museum, has kindly given me the following note on this subject:

"Evidence is accumulating to suggest that there is a cultural break of some magnitude between the more advanced northern tribes of Central Australia (typified by the Arunta) and the natives of the Western Desert, of which the easternmost representatives are the Luritya (Luritcha). This break is not an absolute one, for some customs have travelled ahead of the main body of new ideas. Thus circumcision, subincision, and the tjurunga cults have almost reached the south coast of Australia, whereas others, such as the stone-working practices, have lagged behind. The idea of making polished stone axes, for

[1] The Hermannsburg natives examined comprised both the more eastern Arunta and the western Luritcha. The preponderance of A in the more western natives is further emphasized if the results are examined from the tribal point of view. Of 32 Luritcha, 25 belonged to A and 7 to O. Of 26 Arunta, 17 belonged to A and 9 to O. Strange to say, amongst 5 natives of mixed Arunta and Luritcha blood, 4 belonged to O and only 1 to A. A doubtful Arunta was A. A man of the Ilpara tribe was A. Of 5 natives tribally not allocated, 4 were O and 1 was A.

example, has not passed far over this break, although traded examples have occasionally been seen to the west.

"Schmidt (8) has shown that this break is well marked in the case of the languages; the Arunta belonging to his Northern Australian language group, whereas the Luritya belong to the more primitive Southern group.

"The social organization of these tribes also seems to indicate the same cultural break, and to suggest that the Luritya classificatory system is an attempt to adapt and combine some features of the organization of the Western Desert peoples with that of the Arunta four-class system.

"The natives, both Arunta and Luritya, seem to recognize that the Arunta are the more advanced people, and in the zone of contact between the two tribes the expression 'Luritya' is frequently employed in a contemptuous sense.

"In a broader way the reality of these cultural differences is supported by the data derived from physical measurements, and the analysis of some data, now being attempted, seems to indicate that the percentage of short-statured mesocephalic and wavy to curly-bearded natives present in the population becomes higher as one proceeds southward, whereas the tall, long-headed, and straighter-haired natives become relatively more abundant as one approaches the north coast.

"Dixon (4), working on other data, has also shown that the percentage of distribution of certain cranial characters separates the northern natives from the more primitive peoples of the south."

III. DISCUSSION OF THE RESULTS.

Our data have been rearranged so that on tabulation the results are clear and anomalies at once recognizable. Seventy of the natives examined were pure-bloods, of whom 46 belonged to Group A and 24 to Group O. No. 34, a half-caste, is not included in this seventy, but appears in the tables (he belonged to Group O). All these tests with key sera II and III were perfectly clear. The data have been arranged in four tables, showing the results of the testings carried out between each group of native red cells and the sera of its own and of the other group. In the first two tables appear also the results of the original blood-grouping with the key sera of European origin, as well as the results of testing the native sera with three sets of European red cells belonging to Groups A and B (two samples). The reactions between native and European bloods are separated in the tables by two heavy lines at right angles from the reactions between native cells and native sera. The tests between native and European bloods numbered 342, and those between native cells and native sera 293.

The 142 tests between native red cells and Key Sera II and III call for no comment. These are given in the vertical columns in Tables 1 and 2. Two

hundred tests were carried out between European red cells belonging to Groups A and B and native sera. These tests (Table 2) with native sera containing both the agglutinins a and b were all positive, as they should be, though it will be noticed that in four instances the positive result with Group B red cells is described as slow in appearing, or as being less marked compared with the results of red cells of Group A against the same serum. This suggests that, though the native sera contain b, this agglutinin is sometimes present in less amount than agglutinin a in sera containing both. In Table 1 there are again instances (6 in number) in which the agglutinin b in the native serum seems to exert a weaker effect on European red cells containing B than might be expected. In Table 1 we see that in 45 tests between European red cells A and native sera b, i.e., between red cells and sera of the same group, instead of all the results being pure negatives, only 41 are such, three showing a trace of clumping though classed as negative, and one being classed as positive though the reaction was slow and the clumping finally less coarse than is usual. The sera concerned are Nos. 41 and 42, which acted similarly on certain native red cells of Group A, and Nos. 63 and 64, which were not tested against native red cells.

The 75 tests between native red cells A and native sera ab, were, as they should be, all positive (Table 2).

The 25 tests between native red cells O and native sera ab were, as they should be, all negative (Table 4).

In Table 1 it is seen that whilst 51 out of 58 tests between native red cells O and native sera b were negative, as they should be, 3 were classed as positive, though the clumping was less intense and slower than normal, and 4 though classed as negative showed a trace of clumping.

Similarly in Table 3, whilst 117 out of 135 tests between native red cells A and native sera b were negative, 5 were positive, as in the cases in Table 1, and 13 showed a trace of clumping.

We find on examining further that only certain sera are concerned in these anomalous reactions with European red cells A and native red cells A and O. Serum 3 shows 3 tests with traces of clumping, and 12 negative; serum 13 shows 1 with a trace and 6 negative; serum 30 has 7 with traces against only 4 negatives; serum 41 has 2 with traces (one with the European cells) and 10 negatives; serum 42 has 9 positives (including the European red cells), 5 with traces, and 9 negative; and sera 63 and 64 show a trace of clumping with the European red cells, not being tested against native red cells. The sera containing these minor agglutinins all belong to Group II containing b. With sera containing ab these minor agglutinins, if present in any case, would be masked when tested against red cells containing A, unless the agglutinin a had been previously absorbed and removed. This could not be done under the field conditions of the trip, all of the 75 positive tests between the native red cells A

and native sera ab not being further investigated. In Table 4 25 tests between native red cells O and native sera ab were all negative; Table 1 shows that minor agglutinable substances may be present in native red cells of constitution O, but owing to the small number of these tests, 25 in all, we have no right to infer in consequence of these results that minor agglutinins are never present in native sera containing both a and b.

Turning again to Tables 1 and 3, we find that 7 native sera showed the presence of minor agglutinins. Two of these (Nos. 63 and 64) were only tested against European red cells A, and need not be further considered. Of the remaining five, 2 (Nos. 30 and 42) met with agglutinable substances in native red cells both of Group A and of Group O. In the case of No. 42, reactions were obtained with some native red cells which were sufficiently marked to be considered as positive, and a slow positive was also obtained with European red cells A. No. 30 when reacting showed only traces of clumping, and gave a negative result with European red cells A. No. 41, in one instance out of four, showed a trace of clumping with native red cells O, and no reaction with 7 samples of native red cells A, though a trace was found with European red cells A, showing that it could be grouped with Nos. 30 and 42 as affecting some red cells of both these groups. Of the two remaining anomalous sera, No. 3 gave traces with 3 out of 11 examples of native red cells of Group A, but was only tested against three samples of native red cells of Group O. If more of the latter had been tested reactions might have been found. No. 13 gave a trace with one out of two sets of native red cells O, and no reaction with four sets of native red cells A and with European red cells A. Again, testing of further samples of these latter might have yielded some evidence of clumping. Thus of the seven sera yielding anomalous results, six gave reactions with red cells containing A, and four with red cells of constitution O. In three cases both types of red cells were affected. In three A cells only were affected, and in one O cells only, but the testings against the non-reacting types were too few to make such failure of significance. It is clear that in some native sera of constitution b minor agglutinins are present capable of acting on minor agglutinable substances in native red cells of constitution O, and in native and European red cells of constitution A. It is possible that all the seven sera concerned had this capacity, though it was only proved in the case of three. It is possible, however, that in one serum (No. 3) the agglutinin could only find a corresponding agglutinable substance in red cells of its own major group A.

In a recent paper Landsteiner (6) in America has given a number of examples of irregular agglutinins, most of which were active only at low temperatures, and usually gave very weak agglutination at room temperature (20°C.). A small number were intermediate between cold agglutinins and

typical iso-agglutinins, so that the reactions were distinctly noticeable under the usual conditions of blood grouping. "In all of the cases with atypically reacting sera the blood cells showed no abnormal behaviour, *and could readily be grouped.*" The irregularities, he found, were superimposed upon the more prominent group properties, and were "not in conflict with the scheme of the four blood groups, the usefulness of which cannot be questioned . . . . In Groups A and AB two types of agglutinins were found in general for cells of one or the other sub-group of Group A; those agglutinating blood AA^2 showed in addition a specific action on cells of group O. The properties of these sera demonstrate again quite definitely the existence of sub-groups in Group A, a distinction hardly significant at present for practical purposes." It would seem then that Blood AA^2 is probably present in our aboriginal population. It is even possible that AA^1 is present also, and if so further testings should eventually reveal it. It is of unusual interest to find in such a long isolated race as the Australian aboriginal minor similarities of this kind to the other races of mankind. It shows that one at least of these minor groups is of great antiquity, and not of comparatively recent acquirement.

SUMMARY.

1. The results of 70 further tests on pure-blood aboriginals in Central Australia showed that 46 belonged to Group A and 24 to Group O.

2. A total of 296 groupings of aboriginals in Central and South Australia show that 173 belonged to Group A and 123 to Group O.

3. Natives in the western part of Central Australia, along the East-West railway line and near the Great Australian Bight, show a preponderance of Group A (123) over Group O (62) of nearly 2 to 1.

A number of natives from Oodnadatta and Alice Springs showed about equal numbers of each, though Group O (30) slightly predominated over Group A (27). In the N.E. corner of South Australia Group A (16) was slightly in excess of Group O (12).

A few tests at Point Macleay, on Lake Alexandrina, in South Australia, showed Group O (13) much exceeding Group A (3).

4. Though the figures are too small to lay much stress upon them, the results shown under (3) may support a view, already put forward on cultural and other grounds, that two hordes, one coming down the west coast of Australia, the other down the east coast, may have met in South and Central Australia.

5. It is suggested that, from the blood-grouping so far done on Australian aboriginals, there is no reason to consider them as other than a pure race, of blood constitution A and O, the occurrence of some B in N.E. Australia being attributed to recent intermixture from neighbouring races.

6. The results referred to under (3) and (4) can be explained by the descendants of a single immigration having split in two and passed down the west coast and the east coast respectively, the western horde in the course of time having developed a considerable proportion of A over O, the eastern horde having developed more or less equal proportions of each, or perhaps a preponderance of O. The two hordes have now made contact in Central and South Australia.

7. Minor agglutinins have been detected in some native sera of constitution b which can affect certain native red cells of constitution A and O.

8. Some of these minor agglutinins can also affect European red cells A.

9. These minor agglutinins seem of the same nature as those met with in other races of men. The finding of these in a race so long isolated as the Australian aboriginal indicates that one at least of such minor agglutinins is of great antiquity in the human race.

REFERENCES.

1. Cleland, J. B.: "Blood-grouping of Australian Aboriginals," Aust. Journ. Exper. Biol. and Med. Sci., 3 (1926), p. 33.
2. Cleland, J. B.: "Blood-grouping of Australian Aboriginals at Oodnadatta and Alice Springs," Trans. Roy. Soc. of S. Aust., 51 (1927), p. 78.
3. Cleland, J. B. and Woollard, H. H.: "Further Results in the Blood-grouping of South Australian Aborigines," Med. Journ. of Aust., 2 (1929), p. 2.
4. Dixon, R. B.: "The Racial History of Man" (1923).
5. Dyke, S. C.: "Determination of Compatability in Bloods," The Lancet (Oct. 29, 1927), p. 900.
6. Landsteiner, K. and Levine, P.: "An Isoagglutinin Reaction of Human Blood other than those Defining the Blood Groups," Journ. of Immunol., 17 (1929), p. 1.
7. Lee, H. K.: "Blood Groups of North Queensland Aborigines with a Statistical Collection of some Published Figures for Various Races," Med. Journ. of Aust., 2 (1926), p. 401.
8. Schmidt: "Die Gliederung der Australischen Sprachen," Wien (1919), map.
9. Tebbutt, A. H.: "Comparative Iso-agglutinin Index of Australian Aborigines and Australians," Med. Journ. of Aust., 2 (1923), p. 346.
10. Tebbutt, A. H. and McConnell, S. V.: "On Iso-agglutinins with a Note on their Distribution among some Australian Aborigines," Med. Journ. of Aust., 1 (1922), p. 201.
11. Woollard, H. and Cleland, J. B.: "Anthropology and Blood-grouping, with Special Reference to the Australian Aborigines," Man, 29 (1929), No. 145.

"BLACK-HEART" OF APRICOTS IN VICTORIA

by

GWENDOLYN M. CHENEY

(Government Research Scholar, Botanical Department,
University of Melbourne).

(Submitted for publication 5th May, 1930.)

A disease affecting apricot trees in Victoria was submitted for investigation in March, 1929. The diseased material was taken from trees several years old, growing at Caulfield, Victoria. The disease was characterized by the wilting and ultimate death of successive branches of the tree, and by dark, discoloured areas seen in the wood when cut.

SYMPTOMS OF THE DISEASE.

The first outward manifestation of the disease in the tree may be observed a few weeks after infection, but it is not until a year later that it presents marked symptoms. In the summer of the first year leaves at the base of certain branches begin to wilt, and finally turn yellow and fall. This continues about half-way up the branch. The bases of the branches affected become bare within a few days of the commencement of wilting. No further effect is observed during the season, except the wilting and falling of an occasional leaf, and unless the branches have been markedly denuded the trees present a healthy appearance, continuing to produce new growth throughout the summer months. During the following winter the flower and leaf buds remain normal. At the end of August, or the beginning of September, when the leaf buds should swell, those on the diseased branches become chaffy and fall off. This symptom is the first to be observed if the wilting of the leaves has been missed in the previous year. The branches at this time are still alive, so that the failure of the buds to develop is the only external indication of the disease. The buds may be affected over a great length of stem, and as a consequence the leaves fail to develop, causing death of the branch. It is occasionally found that the buds on a diseased branch may flower and form fruit, but if this does occur the fruit will sooner or later wither and fall off. In the following year the disease will spread to adjoining branches, and so work round the tree until the whole is involved. The disease ultimately kills the tree, but the time taken for this to occur may vary.

On cutting branches of a diseased tree a brown discolouration is found in the wood, and when the tree is removed from the soil this discolouration can be followed down into the main trunk. Gummosis does not accompany the disease to any extent.

ISOLATION OF THE PATHOGEN.

Twigs from branches of diseased material were selected, which, though showing the characteristic brown stain in the wood, were still living. Sections of these were planted on nutrient media to endeavour to obtain the disease-producing organism in artificial culture. The twigs were sterilized in 1 : 1000 mercuric chloride, and washed in sterile water. The bark was then peeled off with a scalpel sterilized by heat, and sections taken of the stem. These were set out on plates of malt agar and incubated. After four days a white fungal colony had been formed from eleven of the thirteen inoculations, the remaining producing colonies of *Penicillium*. The white colonies were subcultured on plain agar, to remove bacterial contamination, and then transferred back again to malt agar slopes. Six days after inoculation all of the slopes showed, in the white mycelium, small black patches developed in the portion submerged in the medium. A microscopical examination showed a verticillately branched form bearing minute, hyaline conidia, with a production of dark microsclerotia. By means of these diagnostic features the fungus was found to be a species of *Verticillium*, and the form corresponded identically with that described by Czarnecki (2) as the pathogen causing "black-heart" disease of apricots in California. The symptoms of the disease described by Czarnecki agree in many respects with those shown by the diseased trees in Victoria. The same wilting of successive branches is observed, and the characteristic brown stain in the wood. Czarnecki, however, states that wilting commences at the tip of a branch, working downwards, but this has not been observed in any case in Victoria, where the wilting commences at the base of the branch, working upwards.

Ewart (4) found that the channel of ascent of sap was nearer the periphery of the wood in the older parts of a branch, that the central wood vessels were also concerned in the ascent in the young parts of the branch, and that if the younger wood was killed by a poisonous solution the path of the ascent of sap could be transferred to the older wood. The leaves at the apex of a branch will therefore receive their vascular supply by way of the central wood vessels, while those nearer the base will receive a supply of sap from vessels nearer the periphery.

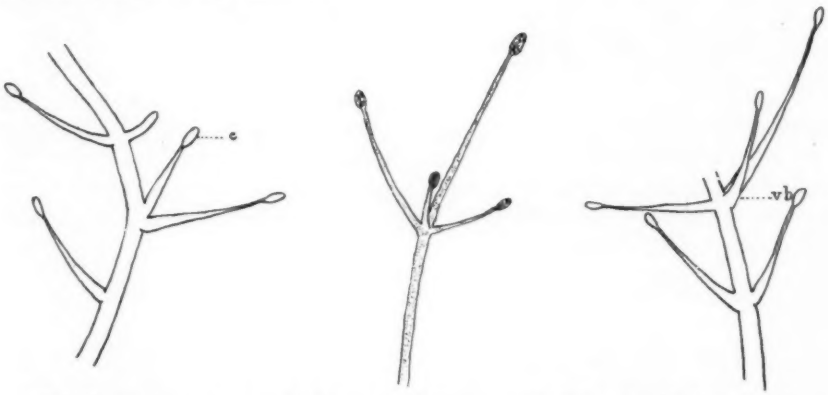
If a section is taken across the base of a branch of a diseased apricot tree, the brown stain denoting the gummy substance deposited in the vessels is seen in the outer parts of the wood cylinder. Therefore it seems evident

from Ewart's results that this affords an explanation for the wilting of the basal leaves of a branch before the upper leaves, as it is the peripheral vessels supplying the lower leaves which become blocked at first.

THE FUNGUS.

The colonies are regular, and approximately spherical. The main growth of the mycelium is submerged, with a tuft of white aerial mycelium in the centre. The characteristic feature of the fungus in culture is the production of the small, dark sclerotia. These are developed more thickly in definite zones, giving the appearance of rings in the medium. (Plate 1.)

Microscopically the fungus shows very numerous, small, elliptical conidia, $4{\cdot}5 - 6 \times 3{\cdot}5 - 4\mu$, being formed at the tips of verticillate branches of the septate hyphae. (Text figure 1.)

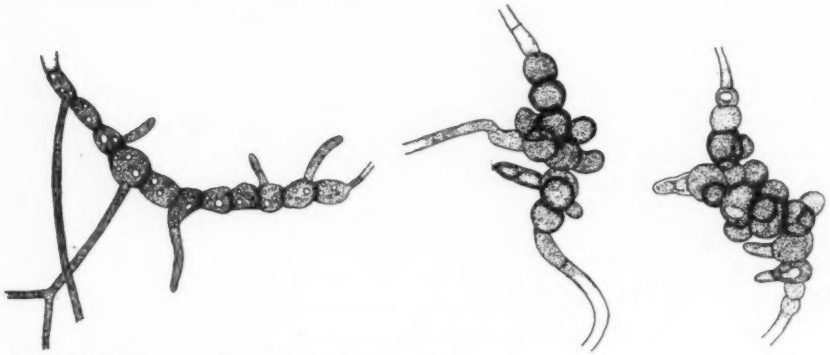


Text figure 1. a, b, c, Production of conidia on verticillately branched conidiophores. × 625. c=conidium; vb=verticillate branching.

On becoming free from the tips of the branches the conidia adhere together in small masses. In five or six days microsclerotes are formed on the mycelium. Dark vacuolated cells appear along the hyphae, and secondary cells of the same nature are produced from them, forming a sclerotial mass 40–80μ in length. These become more numerous and darker as the colonies grow older, until finally the dark colouration due to their formation spreads through the medium, and little aerial growth is obvious. In cultures several months old patches of new white aerial hyphae are sometimes produced. (Text figure 2.)

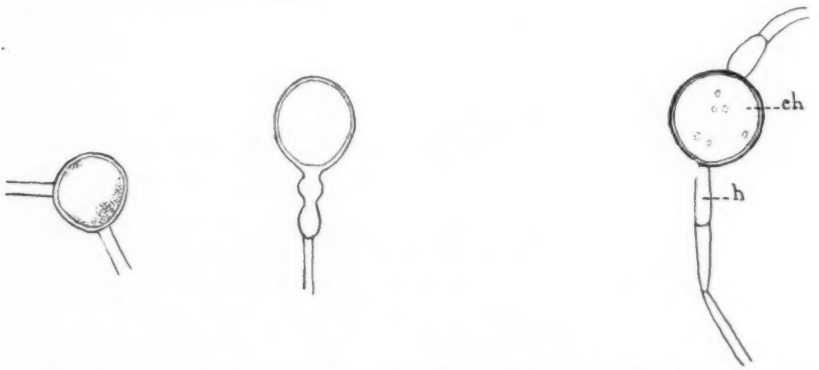
To determine whether the nature of the fungus remains the same on media of different composition, it was also cultured on Coon's, ground maize, and Lemco-peptone agars. On the ground maize agar, which is a very rich medium, there was a relatively greater development of the white aerial mycelium.

Coon's agar proved an excellent medium for culture of the fungus, the zoning of the sclerotes being particularly well marked.



Text figure 2. a, b, c, Microsclerotia. × 625.

An examination of the white aerial hyphae from the cultures showed, in addition to the conidia, swellings along the length of the hyphae (chlamydospores). These were large hyaline structures, varying a little in shape, but mainly spherical, and containing oil drops. They were developed either at the ends of hyphae or in an intercalary position, and on the same hyphae as were producing conidia. (Text figure 3.)



Text figure 3. a, b, c, Structures resembling chlamydospores, which are found in addition to conidia and microsclerotes. × 625. ch=chlamydospore; h=hypha.

Diseased twigs of the apricot were placed in a moist atmosphere and left for several weeks. On examination they were found to have produced white mycelium from the cut ends of the twigs and from the region of the buds.

This was seen to be made up of the verticillately branched conidiophores, bearing conidia.

Germination of the conidia in dilute turnip extract was observed. One, or sometimes two, germ tubes were first formed from the conidium. These tubes later branched profusely, developing into a fine mycelium.

The fungus isolated by Czarnecki (2) from apricots in California agrees in all respects with the form isolated in Victoria. Although the American isolation was recorded as a *Verticillium*, no attempt was made to place it specifically. Since then a similar fungus has been reported on apricots from France and Canada.

SYSTEMATIC POSITION OF THE FUNGUS.

The characters of the fungus observed in culture indicate that the species is identical with the microsclerote-producing form described by Van der Meer (8) as *Verticillium dahliae* (Kleb.). Van der Meer states that *V. dahliae* differs from *V. albo-atrum* (Reinke and Berthold) chiefly in the mode of production of the black microsclerotia. Lateral cells are given off from the dark spherical cells formed along the mycelium in *V. dahliae*, giving rise to sclerotia measuring 40–80μ, whereas no lateral cells are formed in *V. albo-atrum*. Rudolph (6), investigating a tomato wilt in California, ascribes the disease to *V. albo-atrum*, and states that the fungus is identical with that causing "black-heart" of apricots, described by Czarnecki (2). Van der Meer, however, classifies the *Verticillium* isolated by Czarnecki from apricots as *V. dahliae*, and Dufrenoy (3) places the form which he has isolated from various plants, including apricots, in the same species.

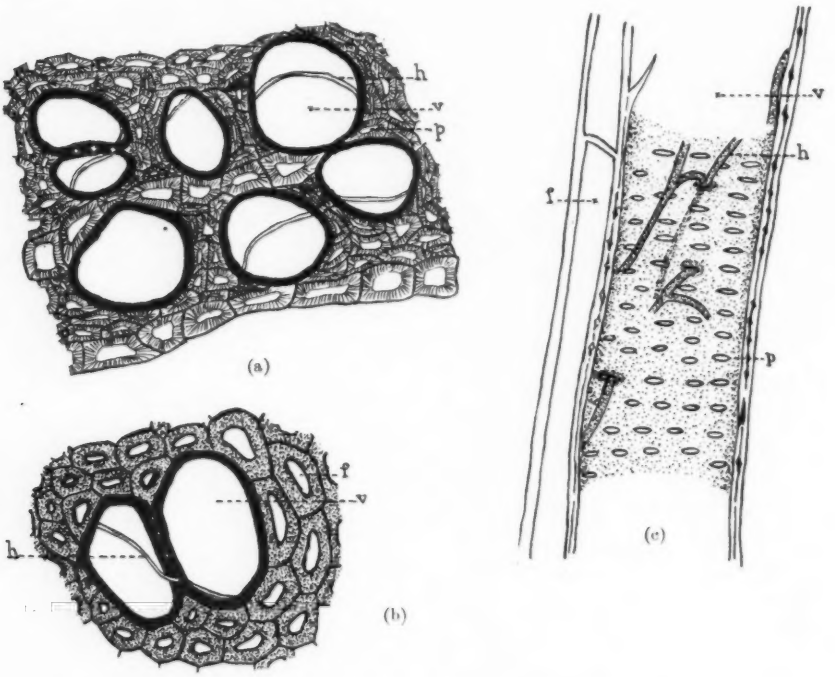
In a paper which has recently appeared, Wollenweber (10) states that he has compared cultures of the *Verticillium* isolated from various hosts. Most of the cultures were considered to be specifically identical with *V. albo-atrum* (Reinke and Berthold). The form described by Van der Meer as *V. dahliae* was also considered morphologically indistinguishable from *V. albo-atrum*. Names have been given to the various strains, but all are classed in the species *V. albo-atrum*.

HISTOLOGY OF DISEASED STEMS AND ROOTS.

Transverse sections of the twigs show the presence of a brown gummy substance in the affected parts, particularly in the medullary rays, and in the older wood vessels, which are blocked by it. Parts of a young diseased stem were kept for a time in hydrofluoric acid to soften the woody tissues. These were then imbedded in paraffin, and microtome sections were made. They were stained by a method which demonstrates fungal hyphae in plant tissues, using

a saturated solution of methylene blue in absolute alcohol, followed by a saturated solution of eosin in clove oil. Fungal hyphae could be seen clearly in transverse section crossing the younger wood vessels. They appeared to pass from one vessel to another through pits on the side walls.

Longitudinal sections stained by the same method also demonstrated clearly the presence of the hyphae in the wood and their passage from one vessel to another through the pits. A few vessels, containing the gummy substance which is produced characteristically in the older affected tissues, were packed tightly with closely wefted hyphae. (Text figure 4.)



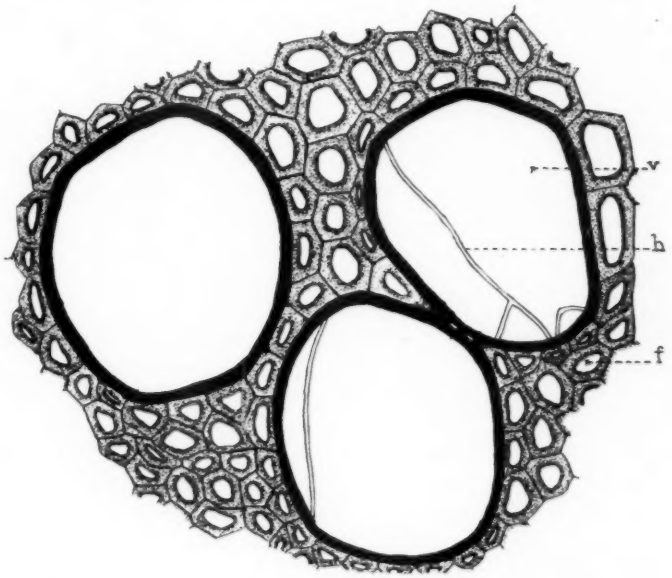
Text figure 4. Sections of diseased stems showing fungal hyphae in the wood.

(a) Transverse section of the wood with hyphae traversing the vessels. × 103.

(b) Hypha passing through a pit in the wall of the vessel. × 625.

(c) Longitudinal section of a vessel with hyphae passing through the pits. × 625. h= hypha; v=vessel; p=pit; f=fibre.

Microtome and hand sections were made of lateral roots from young diseased trees. These were stained with cotton blue. Though showing no brown discolouration when observed with the naked eye, in section many of the wood vessels were found to contain the blocking substance. Very fine hyphae, which had taken up the blue stain, could be seen traversing the vessels.



Text figure 5. Section of a diseased root showing hyphae in the vessels. v=vessel; h=hypha; f=fibre.

A method described by Cartwright (1) for staining hyphae in the tissues (safranin followed by picro-aniline blue) was also used. By this method the finest of hyphae in very small rootlets could be clearly demonstrated. The hyphae were seen to branch profusely, passing into every wood fibre as well as into the vessels. (Text figure 5.)

INOCULATION EXPERIMENTS.

Six two-year-old apricot trees, of the varieties Mansfield and St. Ambrose, were transplanted during the winter and allowed to become firmly established. On September 16, when the leaf buds were unfolding, inoculations were carried out on two of these trees. Six-day-old cultures of *Verticillium* on ground maize agar were used. Three young branches of each of these trees were selected, and washed near the base with 1:1000 mercuric chloride and then sterile water.

With a sterile scalpel an incision was made through the bark, into the wood, in such a manner that the cut tissue formed a flap. Under this was inserted part of the active mycelium. Cotton wool soaked in sterile water was then wrapped round the stem at the point of the incision, and the whole covered with oiled silk and bound. The same operation was performed on a third tree, with the exception of the insertion of the fungus, for use as a control. After four weeks an inoculated branch was removed from one of the trees, and cultures made from sections. A growth of *Verticillium* was obtained from sections taken not more than an inch above or below the inoculation. Though reisolation of the fungus from the branches was accomplished, a careful observation of these inoculated trees throughout the following spring and summer months failed to show any symptoms of disease, and the trees made vigorous and healthy growth.

A further means of reproducing the disease by inoculation with the fungus was therefore sought, and it was thought possible, since many species of *Verticillium* are inhabitants of the soil, that this disease-producing form might enter by way of the roots.

Another of the trees was selected for root inoculation. A suspension of spores from a pure colony of *Verticillium* was made in sterile water. The soil was carefully removed from around the base of the tree, until the uppermost roots were exposed. The spore suspension was then poured on the roots, and the soil replaced. This inoculation was carried out on October 7, and on December 19, that is, two months after inoculation, the tree began to show signs of disease. Leaves at the base of three branches began to wilt and turn yellow, finally dropping off. This continued gradually, until all the leaves on the lower half of the branch had fallen. No further manifestation of the disease was seen, and the tree continued to grow. This reproduction of the symptoms of the disease indicated that the fungus entered the tree through the soil, and consequently, to prove this, a second tree was treated in the same way on December 19. On February 3, 1930, exactly similar symptoms were seen on the second tree. The leaves at the base of the branches wilted and fell, until half the branch was involved. In this case the effect was more marked than before, because during December and January the tree had made rapid growth, so that the branches were much longer, and the falling of half the leaves left them more noticeably bare. A branch was removed from this tree. Sections were taken from the basal and apical parts of the branch, as well as from intermediate regions. These were treated in the usual way and planted on malt agar. From all the sections, with the exception of those bearing contaminations of *Penicillium*, a growth of the *Verticillium* was produced.

These results definitely prove that the pathological symptoms were caused by the *Verticillium*, which gained access to the tree through its roots, the interval between inoculation and wilting being six to eight weeks. (Plate 2.)

SALTATION OF THE FUNGUS IN ARTIFICIAL CULTURE.

A large plate of ground maize agar was inoculated in nine places from a pure colony of *Verticillium* growing on a malt slope. This was carried out with the intention of obtaining mycelium with which to inoculate the apricot trees during the study of the disease. It became apparent, however, after a few days that some of the colonies on this plate were not normal. Four of the colonies after eleven days showed no sign of sclerotes, which should normally have been produced in five to six days, but they had an abnormal development of white aerial mycelium covering the whole colony. One colony showed sclerote production only on one half, while the remaining four, though more normal, showed indications of sectoring, that is, some sectors showed sclerotes while others did not. (Plate 3.)

The abnormal form without sclerotes was thought to be a saltant from the normal *Verticillium* type, and consequently attempts were made to prove this. The slope from which the inocula producing the saltants were taken bore an old colony which had a dense black crust of sclerotia on the surface, but appeared in no way different from a normal old colony. Subcultures were made from saltants on to plates of ground maize agar, and apparently pure cultures of the saltant were obtained. (Plate 4.)

The form was examined microscopically, and found to produce conidia on verticillate branches, the form being morphologically identical with the normal one, with the exception of the absence of sclerotes. To ascertain whether the saltant reverted to the normal form when grown on the original medium, inocula were set out on malt plates. No sclerotes were produced, though the colonies were examined after several weeks. The saltant did not make such good growth, however, or produce such an abundance of aerial mycelium as when grown on ground maize agar, which seems to be an ideal medium for this purpose.

To demonstrate the saltant character of this new form conclusively, and to eliminate any chance of a mixed inoculum, single spore colonies were obtained from the original malt slope on plain agar. One single spore colony was transferred to each of a number of malt and ground maize agar plates, and the saltant appeared in some of these colonies, developing from a single spore.

SUMMARY.

1. A disease of apricot trees in Victoria, characterized by wilting of the leaves and subsequent death of successive branches of the tree, was investigated.

2. A species of *Verticillium*, thought by Van der Meer to be *V. dahliae*, but considered as a strain of *V. albo-atrum* by Wollenweber, was isolated from diseased branches. The fungus in artificial culture produced small, hyaline conidia at the tips of verticillate branches, dark microsclerotia, and structures resembling chlamydospores.

3. In the tissues of the host the mycelium was found in the younger wood vessels, the hyphae passing from one vessel to another through the pits.

Characteristic of the disease is the production of a brown, gummy substance in the older wood vessels. This discolouration can be seen macroscopically, and is one of the symptoms of the disease.

4. Young apricot trees were inoculated by introduction of the mycelium into the branches. No symptoms of disease were found as a result. Inoculation of the roots with a spore suspension, however, produced symptoms of the disease in six to eight weeks. This experiment was performed on two trees, and both contracted the disease, showing that the pathogen enters by way of the roots. Sections were taken from a branch of a tree showing symptoms of disease produced in this manner, and these gave a growth of *Verticillium* in culture.

5. Prolonged culture of the fungus on artificial media produced a saltant, which differed from the normal form in the absence of microsclerotia.

I wish to thank Dr. McLennan for her untiring help during the carrying out of the investigation, and Professor Ewart for his interest in the work and his many helpful suggestions. I am indebted to Mr. T. Kilvington for the photographic illustrations.

REFERENCES.

1. Cartwright, K. St. G.: "A Satisfactory Method of Staining Fungal Mycelium in Wood Sections," Ann. Bot., 43 (1929), p. 412; Abs. Stain Technology, 4 (1929).
2. Czarnecki, Helen: "Studies in the So-called 'Black-heart' Disease of Apricot," Phytopath., 13 (1923), pp. 216-224.
3. Dufrénoy, J. et M. L.: "Hadromycoses," Ann. des Epiphyties, 13 (1927), pp. 195-212.
4. Ewart, A. J.: "The Ascent of Water in Trees" (second paper), Proc. Roy. Soc., B. 79 (1907), pp. 34-396.
5. Rankin, W. H.: "Thrombotic Disease of Maple" (Abs.), Phytopath., 4 (1914), pp. 395-396.
6. Rudolph, B. A.: "*Verticillium* Wilt of Tomatoes in California," Phytopath., 16 (1926), p. 234.
7. Sibilia, C.: "Il deperimento degli Albicocchi," Boll. R. Staz. Pat. Veg., 6 (1926), pp. 377-380.
8. Van der Meer, Jikke. H. H.: "*Verticillium* Wilt of Herbaceous and Woody Plants," Med. Landbouwhoogeschool Wageningen, 28 (1925), pp. 1-82.
9. Van der Meer, Jikke. H. H.: *Verticillium* Wilt of Maple and Elm Seedlings in Holland," Phytopath, 16 (1926), pp. 611-614.
10. Wollenweber, H. W.: "Die Wirtelpilz-Welkekrankheit (Verticilliose) von Ulme, Ahorn und Linde usw," Arb. Biol. Reichsant. fur Land- und Forstwirtsch, 17, 3 (1929), pp. 273-299.
11. Zimm, L. A.: "A Wilt Disease of Maples" (Abs.), Phytopath., 8 (1918), pp. 80-81.

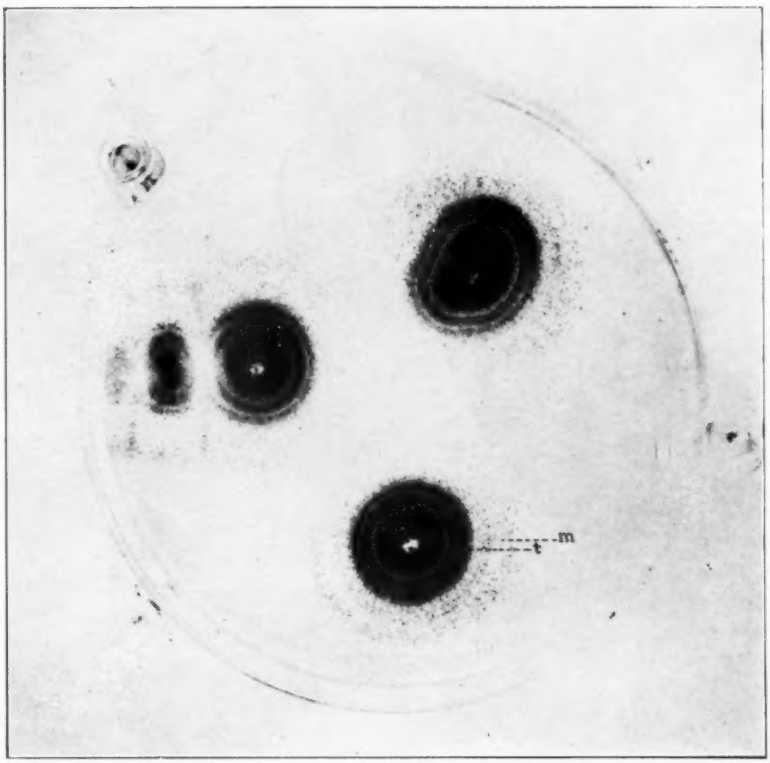


PLATE 1.

Photograph of normal colonies of *Verticillium* growing on Coon's agar. m=microsclerotia; t=tuft of white aerial mycelium.

Characteristic of the disease is the production of a brown, gummy substance in the older wood vessels. This discolouration can be seen macroscopically, and is one of the symptoms of the disease.

4. Young apricot trees were inoculated by introduction of the mycelium into the branches. No symptoms of disease were found as a result. Inoculation of the roots with a spore suspension, however, produced symptoms of the disease in six to eight weeks. This experiment was performed on two trees, and both contracted the disease, showing that the pathogen enters by way of the roots. Sections were taken from a branch of a tree showing symptoms of disease produced in this manner, and these gave a growth of *Verticillium* in culture.

5. Prolonged culture of the fungus on artificial media produced a saltant, which differed from the normal form in the absence of microsclerotia.

I wish to thank Dr. McLennan for her untiring help during the carrying out of the investigation, and Professor Ewart for his interest in the work and his many helpful suggestions. I am indebted to Mr. T. Kilvington for the photographic illustrations.

REFERENCES.

1. Cartwright, K. St. G.: "A Satisfactory Method of Staining Fungal Mycelium in Wood Sections," Ann. Bot., 43 (1929), p. 412; Abs. Stain Technology, 4 (1929).
2. Czarnecki, Helen: "Studies in the So-called 'Black-heart' Disease of Apricot," Phytopath., 13 (1923), pp. 216-224.
3. Dufrénoy, J. et M. L.: "Hadromycoses," Ann. des Epiphyties, 13 (1927), pp. 195-212.
4. Ewart, A. J.: "The Ascent of Water in Trees" (second paper), Proc. Roy. Soc., B. 79 (1907), pp. 34-396.
5. Rankin, W. H.: "Thrombotic Disease of Maple" (Abs.), Phytopath., 4 (1914), pp. 395-396.
6. Rudolph, B. A.: "*Verticillium* Wilt of Tomatoes in California," Phytopath., 16 (1926), p. 234.
7. Sibilia, C.: "Il deperimento degli Albicocchi," Boll. R. Staz. Pat. Veg., 6 (1926), pp. 377-380.
8. Van der Meer, Jikke. H. H.: "*Verticillium* Wilt of Herbaceous and Woody Plants," Med. Landbouwhoogeschool Wageningen, 28 (1925), pp. 1-82.
9. Van der Meer, Jikke. H. H.: *Verticillium* Wilt of Maple and Elm Seedlings in Holland," Phytopath, 16 (1926), pp. 611-614.
10. Wollenweber, H. W.: "Die Wirtelpilz-Welkekrankheit (Verticilliose) von Ulme, Ahorn und Linde usw," Arb. Biol. Reichsant. fur Land- und Forstwirtsch, 17, 3 (1929), pp. 273-299.
11. Zimm, L. A.: "A Wilt Disease of Maples" (Abs.), Phytopath., 8 (1918), pp. 80-81.

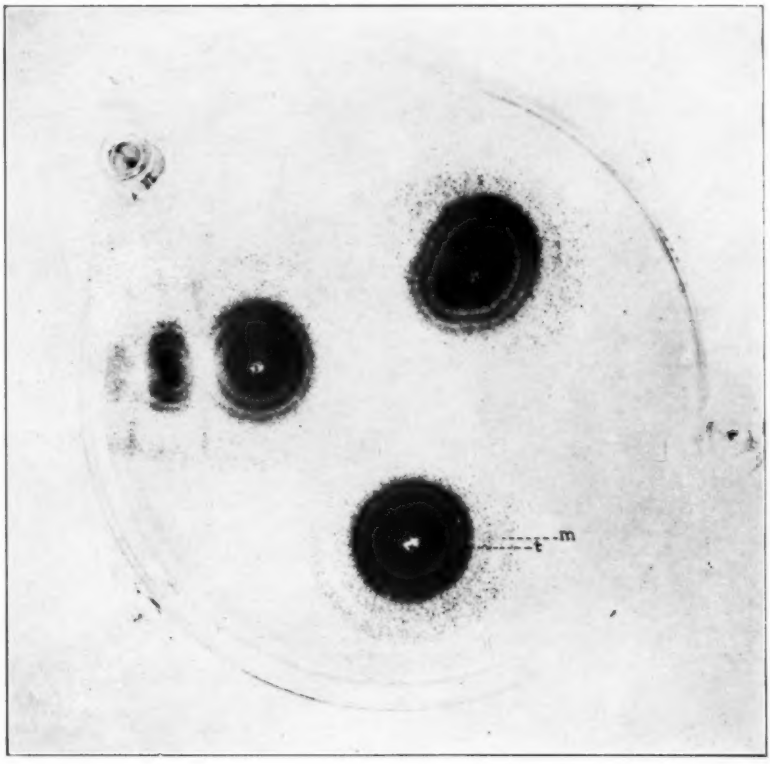


PLATE 1.

Photograph of normal colonies of *Verticillium* growing on Coon's agar. m=microsclerotia; t=tuft of white aerial mycelium.

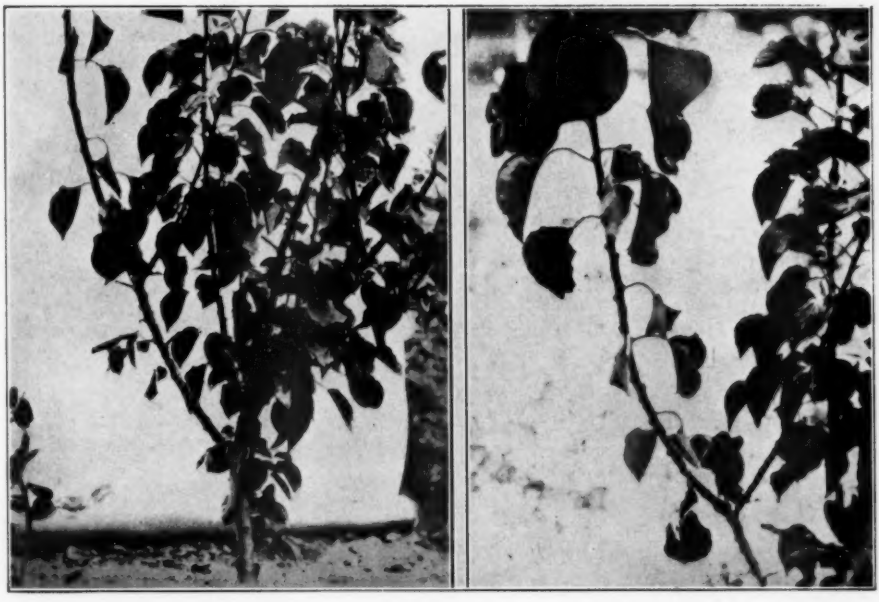


PLATE 2.

(a) Photograph of an inoculated tree showing the wilting of the basal leaves, which is the first symptom of disease.

(b) Photograph of a closer view of the wilting leaves on a branch of the same tree.

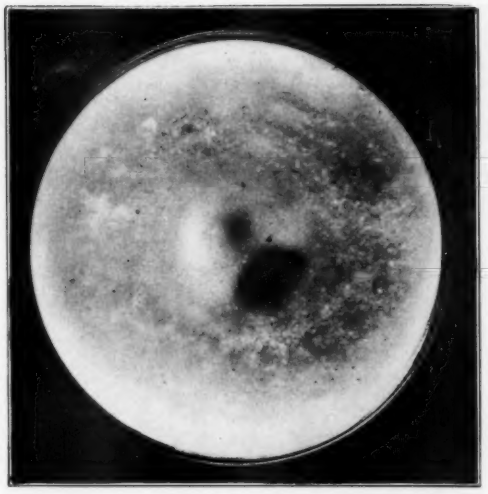


PLATE 3.

Photograph of a sectoring colony growing on ground maize agar. Microsclerotia are being formed only in the dark sector.

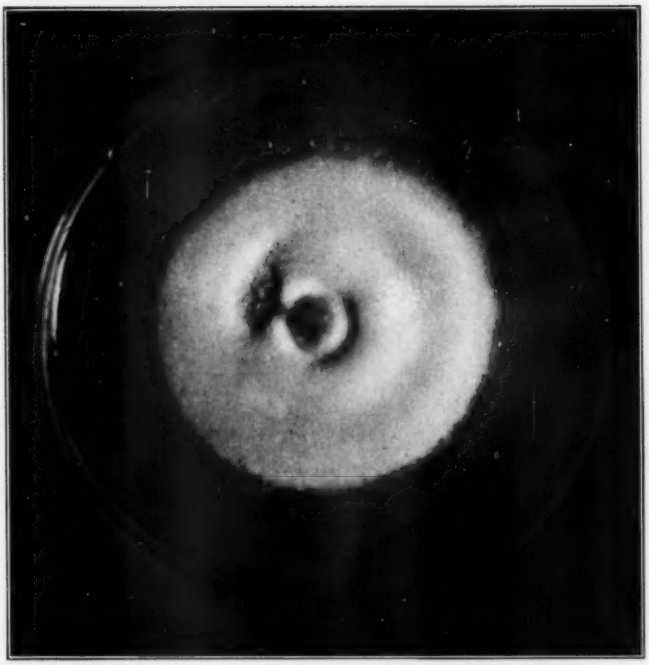


PLATE 4.
Photograph of a saltant produced from a single spore.